Pathophysiological effects of calcitonin gene-related peptide in a model of cardiovascular dysfunction and remodelling

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Pathophysiological effects of calcitonin gene-related peptide in a model of cardiovascular dysfunction and remodelling

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ABSTRACT

Hypertension is the number one killer in the Western world at present and is a major risk factor for the development of cardiovascular disease. However, despite the extensive range of therapies, the mechanisms involved in its onset still remain unclear, therefore there is a need to investigate the mechanisms involved. Calcitonin gene-related peptide (CGRP) is best known as a sensory nerve-derived neuropeptide, a potent microvascular vasodilator and is suggested to be protective in a range of models of hypertension. The aim of this study was to investigate the influence of CGRP on vascular mechanisms in an Angiotensin-II (Ang II) model of hypertension.

Wildtype (WT) and αCGRP knockout (αCGRP KO) mice were infused with either vehicle (saline) or Ang II for 14 (1.1mg/kg/day) or 28 (0.9mg/kg/day) days. Under both normal physiological conditions and after saline infusion, blood pressure (BP) and gross pathological observations did not differ between WT and αCGRP KO mice. Ang II infusion caused an increase in BP in the mild-moderate hypertensive range in WT mice at both 14 and 28 days. However in the absence of αCGRP, this hypertension was exacerbated, this being in the moderate hypertensive range at day 14, and becoming severely hypertensive by day 28.

When measuring CGRP levels, plasma and mRNA αCGRP expression was upregulated in the aorta and mesenteric vessels of hypertensive WTs, and localisation of CGRP (α and β) was visible in endothelial and smooth muscle cells of the aorta. This increase in CGRP expression was also accompanied by an increase in CGRP receptor expression at day 28 in WTs.

Vascular inflammation/remodelling of the aorta was apparent in the developing hypertension, perhaps via the loss of endothelial derived nitric oxide synthase (eNOS) and a resultant increase in NADPH oxidase. This inflammation/remodelling was characterised by increased luminal wall width, collagen expression and cytokines/adhesion molecules. This inflammation/remodelling was exacerbated in Ang II-treated αCGRP KOs.

Abstract

This study provides evidence that i) αCGRP is upregulated at the vascular level in hypertension and ii) deletion of αCGRP is associated with enhanced Ang II-induced hypertension and vascular injury. This therefore suggests a protective role for αCGRP in this model.

For Papa, a true inspiration

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LIST OF ABBREVIATIONS

2K1C	Two-kidney, 1-clip
αCGRP	Alpha calcitonin gene-related peptide
βCGRP	Beta calcitonin gene-related peptide
Ab	Antibody
AC	Adenylate cyclise
ACE	Angiotensin converting enzyme
Ach	Acetylcholine
ADH	Antidiuretic hormone
Ag	Antigen
AM	Adrenomedullin
IMD/AM2	Adrenomedullin-2, Intermedin
Amy	Amylin
Ang II	Angiotensin II
APS	Ammonium persulphate
ARB	Angiotensin receptor blocker
ATP	Adenosine triphosphate
BH_4	(6R)-5, 6, 7, 8-tetrahydro-L-biopterin
BMI	Body mass index
BP	Blood pressure
cAMP	Cyclic adenosine monophosphate
CCM	Cirrhotic cardiomyopathy
cDNA	Complementary DNA

List of Abbreviations

cGMP	Cyclic guanasine monophosphate
CGRP	Calcitonin gene-related peptide
CKD	Chronic kidney disease
CLR	Calcitonin like receptor
CO	Carbon Monoxide
CT	Calcitonin
CVD	Cardiovascular disease
DAB	3.3'-Diaminobenzidine
DNA	Deoxyribonucleic acid
DOCA-salt	Deoxycorticosterone salt
DPI	Diphenyleneiodonium
DRG	Dorsal root ganglion
EC	Endothelial cell
EDHF	Endothelium-derived hyperpolarizing factor
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
EPCs	Endothelial progenitor cells
ERK	Extracellular signal-regulated kinase
ET	Endothelin
GPCR	G protein-coupled receptor
GPX	Glutathione peroxidise
G-SSG-Rd	Glutathione reductase
H_2O_2	Hydrogen peroxide

List of Abbreviations

HO^-	Hydroxyl radicals
HO-1	Heme oxygenase-1
HPRT-1	Hypoxanthine-guanine phosphoribosyltransferase -1
HRP	Horseradish peroxidise
HUVEC	Human umbilical vein endothelial cells
ICAM-1	Intercellular Adhesion Molecule 1
IHC	Immunohistochemistry
IL	Interleukin
IV	Intravenous
K^+	Potassium
KC	Mouse interleukin-8
kDa	Dalton
KO	Knockout
L-Arg	L-arginine
L-NAME	N(G)-Nitro-L-Arginine Methyl Ester
LPS	Lipopolysaccharide
LV	Left ventricle
MAP	Mean arterial pressure
MAPK	Mitogen activated protein kinase pathway
MCP-1	Monocyte chemotactic protein-1
MI	Myocardial infarction
MLA	Monophosphoryl lipid A
mmHg	Millimetre of mercury
mRNA	Messenger ribonucleic acid

List of Abbreviations

MRV	Mesenteric resistance vessels
NaCl	Sodium chloride
NADPH-oxidase	Nicotinamide adenine dinucleotide phosphate-oxidase
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B
NKA	Neurokinin A
NO	Nitric oxide
NOX	NADPH oxidase isoform
NSAIDs	Non-steroidal anti-inflammatory drugs
NTG	Nitroglycerin
$O_2^{\cdot-}$	Superoxide
$ONNO^-$	Peroxynitrite
Ox LDL	Oxidised low density lipoprotein
PACAP	Pituitary adenylate cyclise-activating polypeptide
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGDF	Platelet derived growth factor
PKA	Protein kinase A
PKC	Protein kinase C
PLA_2	Phospholipase A2
Pmol	Picomole
RAMP	Receptor activity modifying protein
RAS	Renin-angiotensin system
RCP	Receptor component protein
RIN	RNA integrity number

List of Abbreviations

RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Reverse transcription
RT-qPCR	Real-time quantitative polymerase chain reaction
SA-HRP	Streptavidin-horseradish peroxidise
SDHA	Succinate dehydrogenase complex, subunit A
SDS	Sodium dodecyl sulphate
SHR	Spontaneously hypertensive rat
SMCs	Smooth muscle cells
SOD	Superoxide dismutase
SP	Substance P
TALH	Thick ascending loop of Henle cells
TEMED	Tetramethylethylenediamine
TG	Transgenic
TGF-β	Transforming growth factor *beta*
TNF-α	Tumor necrosis factor-*alpha*
TPR	Total peripheral resistance
TRPV1	Transient receptor potential cation channel subfamily V member
UV	Ultraviolet
VCAM-1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor
VIP	Vasoactive intestinal peptide
VPR	Volume pressure recording

List of Abbreviations

VSMC	Vascular smooth muscle cell
WT	Wildtype
ZnPP-9	Zinc protoporphrin IX

CHAPTER ONE: INTRODUCTION

1.1 Background

Hypertension is a large economic and public health problem, being a major risk factor for the development of secondary diseases, and contributing to cardiovascular disease, which is the number one killer in the Western world at present. Hypertension is a polygenic disease, involving complex interactions between endogenous mediators and signalling systems (Nagata and Hattori, 2011; Banerjea, 1963). Its high incidence and knowledge that it is a risk factor for the secondary development of heart and renal disease, necessitates a better understanding of molecular causes, in order that treatment may be personalised more precisely than present. Despite a range of therapeutics, mechanisms involved in the onset of hypertension still remain unclear, therefore there is a real need to investigate the factors involved.

The role of vascular smooth muscle in retaining vasodilator tone and thus a healthy blood pressure (BP) is clear. To date, emphasis has been placed on the importance of endothelial cells in influencing vascular tone, as they release essential vasodilator mediators including prostacyclin (Moncada *et al.* 1976), nitric oxide (Furchgott and Zawadzki, 1980) and a range of substances classified as endothelium-derived hyperpolarizing factors (EDHF) (Félétou and Vanhoutte, 2006; Köhler and Hoyer, 2007). However, there are other endogenous vasodilators that can act to directly relax vascular smooth muscle. Some do this in addition to initiating endothelial-dependant relaxing mechanisms, one of these being calcitonin gene-related peptide (CGRP) (Brain and Grant, 2004; Deng and Li, 2005; Wang, 2005; Márquez-Rodas *et al.* 2006). The potent vasodilator activity of CGRP was discovered in 1985 in a study carried out in a range of species including humans (Brain and Williams, 1985). There is strong evidence that CGRP has effects in influencing vascular tone in a range of tissues that include human skin (Brain *et al.* 1986), the kidney (Uddman *et al.* 1986), and the cerebral vasculature (Edvinsson *et al.* 1985).

CGRP is the most potent microvascular vasodilator known to date, also producing positive chronotropic and inotropic effects (Brain *et al.* 1985). Widely expressed in the nervous system, it is known to play an important role in neurogenic

vasodilation in the skin and to increase blood flow in response to injury or noxious stimuli (Brain and Grant, 2004). CGRP is a 37 amino acid neuropeptide which was discovered in 1982 (Amara *et al.* 1982). It is a member of the calcitonin family of peptides which also include adrenomedullin (AM) (Kitamura *et al.* 1993), intermedin (IMD/AM2), (Roh *et al.* 2003) and amylin (Amy), (Westermark *et al.* 1986). Found predominantly in sensory C and Aδ nerve fibres (Goodman and Iversen, 1986), CGRP is considered to play a role in a variety of pathological and physiological processes including vasodilatation, cardioprotection, neurogenic inflammation and pain perception, thus making the receptors for these peptides relevant cardiovascular drug targets (Qi and Hay, 2010).

This introduction will begin by introducing hypertension, the current knowledge of CGRP and its receptors to date, and consider the research carried out previously that implicates a role for CGRP in hypertension, with evaluation of its potential as a therapeutic agent. I will then discuss the aims of this PhD before proceeding on to subsequent chapters.

1.2 Blood pressure regulation

Blood pressure (BP) is known as the pressure exerted by blood on the walls of the arteries (Izzo *et al.* 2008; Herringham and Womack, 1909). Systolic BP is recorded as the highest pressure point; achieved when ventricles are ejecting blood into the vascular system on contraction of the heart. Diastolic pressure is the lowest pressure point, when the heart relaxes following contraction (Oxford Concise Medical Dictionary, 2008).

BP is regulated through several mechanisms, one of which includes the baroreceptor reflex whereby the baroreceptors within high pressure zones such as the aortic arch and carotid sinus detect increases in arterial pressure which in turn signals to the brain. This signal then allows the autonomic nervous system to adjust the pressure through alteration of heart rate and total peripheral resistance to lower BP (Lohmeier and Iliescu, 2011). Baroreceptors which are present in low pressure zones such as the vena cava and pulmonary veins detect low BP. Once detected they can cause a feedback mechanism to regulate the secretion of antidiuretic hormone (ADH/Vasopressin), renin and aldosterone. This results in an increased blood volume and increased cardiac output, therefore an increase in BP (Jacobsen, 1996). The baroreceptor reflex is not a major target in the treatment of hypertension because if blocked, it is thought that individuals may suffer from hypotension and fainting (Lohmeier and Iliescu, 2011).

The renin-angiotensin system (RAS) is another system which controls the long term adjustment of BP via the kidney. During periods of a reduction in BP the kidney detects a loss of blood volume and drop in arterial pressure and compensates for this via activation of the endogenous vasoconstrictor angiotensin II (Ang II) which causes an elevation in mean arterial pressure back to a normal value (Ikonomov and Stoinev, 1979). In contrast to this, if high BP is detected, the steroid hormone aldosterone is released from the adrenal cortex in response to high Ang II or potassium levels. This hormone stimulates sodium retention and potassium excretion by the kidney and results in increased water retention, and thus an increase in arterial pressure (Andersson *et al.* 1972). The RAS is targeted pharmacologically by angiotensin-converting enzyme (ACE) inhibitors and angiotensin receptor antagonists (ARBs). This system and its treatments will be later discussed in more detail.

1.3 Hypertension

In the human cardiac cycle, normal systolic pressure is considered as 115 mmHg and diastolic, 75 mmHg. Hypertension is the chronic elevation of systemic arterial pressure from the accepted normal range, diagnosed when a patient's BP is consistently above 140 mmHg systolic or 90 mmHg diastolic (Izzo *et al.* 2008). However the definition of "normal" may indeed vary depending on a number of factors including sex, age, ethnicity and underlying medical problems. Hypertension can be further defined into 3 categories, depending on severity; mild hypertension is considered as 140-159mmHg systolic, 90-99mmHg diastolic, moderate hypertension; 160-199mmHg systolic, 100-109mmHg diastolic. Severe cases of hypertension display pressure ranges of >200mmHg systolic and >110mmHg diastolic (Reid *et al.* 2001). However diastolic pressure does not need to be high in order to define hypertension, which can be defined as "isolated systolic hypertension" (Staessen *et al.* 1997). This form of hypertension is primarily the result of arterial stiffness (Wallace *et al.* 2007). However when diastolic pressure does increase, there is a greater risk of heart hypertrophy and the progression of arteriosclerosis leading to strokes and heart attacks as diastolic pressure primarily drives the coronary flow (Smulyan and Safar, 2000). Hypertension is in addition to being a major risk factor for the development of secondary diseases, occurs as a consequence of diseases such as obesity and Type II diabetes (Chobanian *et al.* 2003). In Scotland alone last year, the Scottish Health Survey, 2010/11 documented that 20.1% of men and 16.7% of women reported having a cardiovascular condition and/or diabetes in 2010. Multiple health risks in the adult population, were measured using the Scottish Health survey's individual measures of: alcohol consumption outwith the recommended limits, cigarette smoking, not meeting the physical activity recommendations of at least 30 minutes on five or more days week, eating fewer than five portions of fruit and vegetables per day, and having a body mass index (BMI) of 25 kg/m or more (overweight or obese). It is also astonishing to discover that in 2010 just 2% of adults in Scotland had none of these risks, though only 4 % had all five risks. The mean number of risks was 2.7. 59% of adults had three or more risks, while 24% had four or five (conducted by the Scottish Health Survey (*Riaghaltas na h-Alba*), 2010). When factoring in the rest of the UK, one in three adults have high BP, and

nearly half of them do not even realise they have the condition, or are not receiving any treatment (British Heart Foundation 2011).

1.4 Hypertension risk factors

In the present day, the population is highly educated on the risk factors of hypertension due to the high incidence of deaths occurring in the Western world. These risks include stress, tobacco and alcohol use are advertised globally in excess. It is thought that more than 60% of cases occur in people with a BMI greater than 25 (WHO, 2010). In Scotland, prevalence of overweight among adults including obesity (BMI >=25) and morbid obesity (BMI >=30) has continued to increase since 1995 to reach 65.1% and 28.2% respectively in 2010 (Scottish Health Survey, 2010). Certain chronic conditions may result due to high BP induced by obesity including high cholesterol, diabetes and kidney failure. Nevertheless, these are modifiable risk factors and are solely up to the individual to address in order to maintain a healthy lifestyle and reduce their risk of cardiovascular complications in later life.

There are however some risk factors which are unmodifiable and outwith our control which are now discussed in turn.

Age and sex- The incidence of high BP and cardiovascular complications increases proportionally with age in both sexes. It has been stated that over 80% of over 65's are expected to die due to cardiovascular complications (Karavidas *et al.* 2010). Data from the Scottish Health Survey suggest that the prevalence of BP is similar in England and in Scotland. In 2010, 32% of English men and 29% of English women were hypertensive compared to 34% of Scottish men and 31% of Scottish women (British Heart Foundation Statistics Database, 2010). During the ageing process, there is a reduction in endothelial function, which in turn can cause for example, a decline in nitric oxide (NO) production. This has been shown to lead to vasoconstriction, and therefore contributes to the hypertensive phenotype (Yu and Chung, 2001).

Race- High BP and subsequent cardiovascular complications are particularly common among blacks, often developing at an earlier age than it does in whites.

The Health Survey for England in 2006 showed that the proportion of Bangladeshi men with high BP was half that of the general population; in Pakistani and Chinese, the proportion was two thirds of the general population (British Heart Foundation Statistics Database, 2010). Studies have suggested that a deficiency in vitamin D levels could be responsible for the high prevalence of hypertension amongst African Americans (Fiscella *et al.* 2011; Sabanayagam *et al.* 2011). It has also been shown recently that the progression from pre-hypertension to clinical hypertension is accelerated in blacks. In this particular study it was also shown that blacks are predisposed to more severe hypertension than whites (Selassie *et al.* 2011).

Family history and genetic preconditioning- Many forms of cardiovascular disease are hereditary and therefore a genetic contribution is inevitable when considering the "simple" Mendelian traits (Ho *et al.* 2011). In addition, the term “programming” is described as the relationship between early life events and their influences on adult life (Gluckman and Hanson, 2007). Fetal programming by maternal nutritional status is suggested to be a major determinant of adult health and performance (Barker, 2004), and despite previous contradictory debates, there is now epidemiological data to show the relationship of prenatal events to adult onset disease such as Type 2 diabetes, cardiovascular problems, osteoporosis and obesity (Gluckman and Hanson, 2007). The “Life history theory” suggests that during fetal development, increased energy distribution varies between different traits, causing important traits to have larger energy distribution in comparison to minor traits such as tissue repair mechanisms. Thus, alongside with the hypothesis that disease such as stroke and diabetes may show increased adult occurrence due to early life *in utero* maternal undernutrition, this uneven energy distribution in smaller babies may also predispose the individual to be susceptible to disease in later life (Barker, 2004). Gopalakrishnan *et al.* researched the influence of prenatal nutrition and sequential risk of hypertension in adulthood in sheep. No previous studies confirming an association between prenatal nutrient intake and the resulting BP levels in the offspring at adult stages appear to have been published. With methods of dietary intervention, their study showed that maternal nutritional restriction had resulting programmed adjustments in cardiovascular function amongst the offspring as adults. Such

adjustments included increased pre-feeding BP, elevated heart rate at a given mean arterial pressure and increased leptin responses to catecholamine and Ang II (Gopalakrishnan *et al.* 2004).

1.5 Essential and secondary hypertension

Hypertension is classified as either essential (primary) or secondary. Essential hypertension is known to occur spontaneously, with no identified medical cause, accounting for 90–95% of cases. As mentioned in the previous section, there are several risk factors for hypertension which are unavoidable and outwith our control. These include age, sex and race. These factors are of risk to developing essential hypertension. Recently low birth weight and fetal programming has been questioned to also be a risk factor in the development of adult essential hypertension (Uchiyama, 2008) as previously mentioned.

Secondary hypertension is when the elevated BP is occurring as a result of another condition i.e. renal dysfunction, heart disease and tumours, and so results from an identifiable cause. This type is treated differently to the essential hypertension by treating the underlying cause. These include Cushing's syndrome, polycystic kidney disease, pre-eclampsia and adverse effects of certain medications such as non-steroidal anti-inflammatory drugs (NSAIDs) (Uchiyamam *et al.* 2008).

Most of the mechanisms associated with secondary hypertension are fully understood. By comparison, the pathophysiology of essential hypertension is generally less well understood. To date, what is known is that cardiac output is raised early in the disease, together with normal total peripheral resistance (TPR). Over time, this cardiac output drops to normal levels but TPR is increased. There are several theories proposed to explain this. One suggestion is that there is an inability of the kidney to excrete sodium, resulting in natriuretic factors such as atrial natriuretic factor being secreted to promote salt excretion, leading to a side effect of raised TPR and impaired pressure natriuresis due to stimulation of the sympathetic nervous system (Frezza *et al.* 2009 and Pimenta and Oparil, 2009). It is also thought that an overactive renin-angiotensin system leads to vasoconstriction and retention of sodium and water. The subsequent increase in blood volume therefore leads to hypertension (Manrique *et al.* 2009).

1.6 The effects of untreated hypertension

Hypertension occurring over a persistent period of time is associated with the development of a range of secondary cardiovascular diseases, with detrimental consequences such as myocardial infarction (MI), stroke, heart and renal failure, left ventricular hypertrophy (i.e. increased cardiac mass) and stiffening/thickening of the aorta and other major arteries (Branca *et al.* 2007; Susic and Frohlich, 2008). The UK National Institute for Health and Clinical Excellence (NICE) suggests that if those suffering from hypertension in the UK were to have their hypertension controlled (i.e. below 140 mmHg systolic) then there would be a 28–44% reduction in incidence of stroke and a 20–35% reduction in ischemic heart disease (National Institute for Health and Clinical Excellence, 2006). Therefore, treatment of hypertension is clearly important to reduce the morbidity and mortality of these serious cardiovascular events.

If mean arterial pressure (MAP) exceeds 50% above average, a person will have no more than a few months to live unless the condition is treated appropriately (Hall *et al.* 2006). There is no doubt that the constant excessive pressure on the artery walls caused by the increased BP will cause damage to the blood vessels, and additional end organ damage. Therefore, it is fair to say that the higher the BP, and the longer duration without it being controlled, the greater the cardiovascular damage and risk of mortality.

1.7 Methods of treatment for hypertension

Guidelines for the treatment of hypertension have been published in both the UK and US. In the UK, the National Institute for Health and Clinical Excellence (NICE) published in 2007 states that the target goal of treatment is to reduce the BP to a systolic pressure of less than 140 mmHg, and a diastolic pressure of less than 85 mmHg (Williams *et al.* 2004; National Institute for Health and Clinical Excellence, 2006). There are many pharmacological treatments used to try and achieve this e.g. beta-blockers to reduce heart rate, calcium-channel blockers to reduce calcium transport/myocardial contractility, and diuretics which reduce/ inhibit sodium and water retention (Izzo *et al.* 2008). There are also angiotensin converting enzyme (ACE) inhibitors and angiotensin II receptor blockers (ARBs) which target the RAS system as shown in figure 1.1. The effectiveness and choice

of treatments depends on both age and race (Baglioni *et al.* 2007). In addition to this, not all people require drug treatment. There are non-medical ways of reducing BP by simply changing the patient's lifestyle e.g. reducing salt intake, weight loss and exercise. If these recommendations make no difference, then drug treatment is then a recommended option.

Doctors follow a series of guidelines when prescribing anti-hypertensive drugs, as drugs work in different ways for each individual. Below are the recommendations from the National Institute for Health and Clinical Excellence (NICE) which doctors follow when choosing the best anti-hypertensive drug.

- People aged 55 or over, or of African or Caribbean descent, should begin by taking a calcium channel blocker or diuretic.

- Those below the age of 55, and not of African or Caribbean descent, should begin by taking ACE inhibitors. If not suitable then change to an ARB.

- Some patients may require more than one drug in order to reduce and maintain a healthy BP. In cases like this the following guidelines apply;

- If the patient is taking a calcium channel blocker or diuretic and needs additional medication, they should combine this with an ACE inhibitor or ARB.

- If the patient is taking an ACE inhibitor or an ARB and needs additional medication, they should combine this with a calcium channel blocker or a diuretic.

- If the patient needs three drugs to maintain low BP, it is recommended that they take an ACE inhibitor or an ARB, plus a calcium channel blocker, plus a diuretic.

We have now reached a stage where the use of medicines, as described above provides some excellent treatments, but high BP remains a huge problem. Moreover, the molecular basis underlying human essential hypertension still remains unclear. Research into how sensory nerves sense physiological changes and in turn influences BP, cardiovascular and renal function, is providing valuable insights. It is hoped that the elucidation of mechanisms will allow novel pharmacological approaches into the treatment of hypertension to be developed. This will allow us to learn more about the onset of hypertension and also about mechanisms by which we can protect against end organ damage (Brain and Grant, 2004; Wang, 2005).

1.8 The mechanisms involved in hypertension

1.8.1 Angiotensin II and the Renin-Angiotensin System (RAS)

The renin-angiotensin system (RAS) is a system mediated by hormones which regulates BP and water balance. When the volume of blood is low, the kidneys secrete renin, which in turn stimulates angiotensin production. Angiotensin is an oligopeptide present in the blood. It is a hormone and powerful dispogen, derived from the precursor molecule angiotensinogen, a serum globulin produced in the liver (Basso and Terragno, 2001). Angiotensin causes the blood vessels to constrict, therefore resulting in an increase in arterial pressure. Another role of angiotensin is the stimulation of aldosterone secretion from the adrenal cortex. This causes kidney tubules to increase the reabsorption of sodium and water, which in turn also increases BP and therefore induces hypertension, (Solomon *et al.* 2005). The RAS plays a key part in the pathophysiology and development of hypertension and cardiovascular diseases (CVD). During the process of hypertension, vascular remodelling occurs in the small and large arteries, leading to vascular complications and an increasing cardiovascular risk. There is evidence to date that hypertension-induced vascular injury occurs due to inflammation, which has a significant effect on the occurrence of high BP and other vascular complications. Therefore, the RAS is now a major target for developing drugs to reduce BP (Marchesi *et al.* 2008, see figure 1.1). As previously discussed, treatments which target this system include ACE inhibitors and ARBs which currently are effective in the treatment of hypertension. However the worldwide problem of hypertension still remains, despite the large scale use of RAS target treatments.

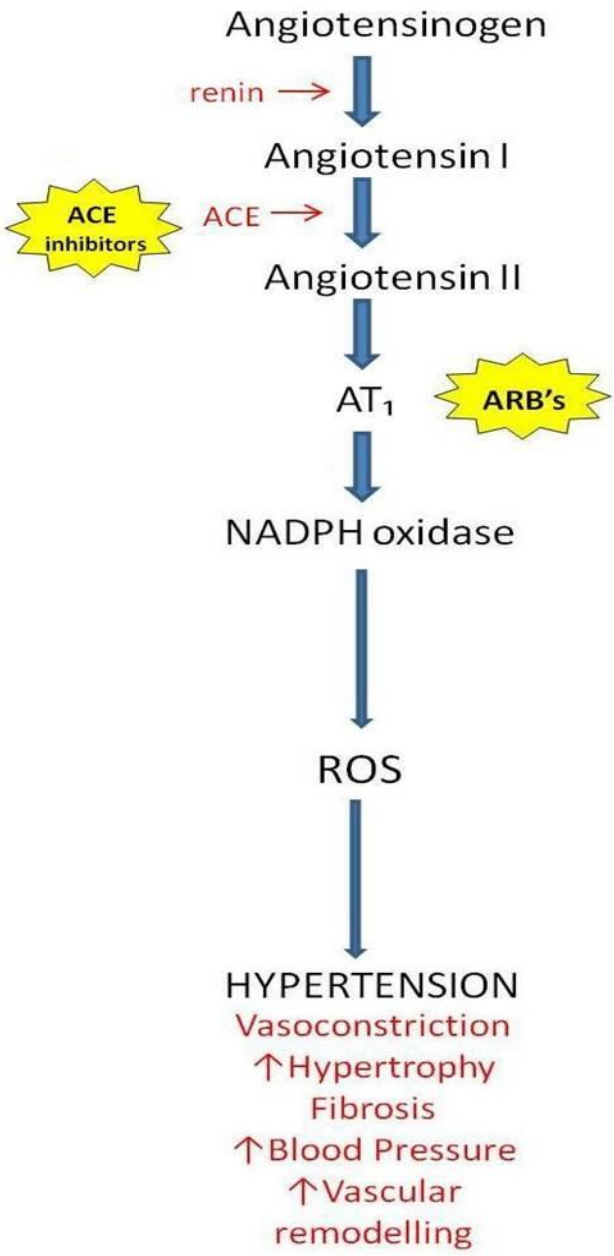


Figure 1.1. Summary of the role of vascular oxidative stress in the pathogenesis of hypertension. Angiotensinogen is converted into angiotensin I by renin. Angiotensin I is then converted by angiotensin converting enzyme (ACE) to angiotensin II (Ang II). This can be blocked via the administration of ACE inhibitors. Ang II then acts on the AT_1 receptor to stimulate nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) synthesis which in turn cause an increase in reactive oxygen species (ROS) generation and therefore the development of hypertension and vascular inflammation/remodelling. Angiotensin receptor blockers (ARBs) are used therapeutically to inhibit angiotensin II binding to the AT_1 receptor. Figure adapted based on that by Platten *et al.* 2009.

1.8.2 Vessel homeostasis

In a healthy individual the normal state of the vessel wall is tightly regulated by a delicate balance between vasoconstriction and vasodilation, in addition to tight regulation of the intracellular pH via sodium and hydrogen exchange (Izzo *et al.* 2008). The contractile state of the VSMCs is the primary determinant of arterial lumen diameter which in turn affects blood pressure and flow. Contraction of the vessel depends primarily on external stimulus and a change in membrane potential and receptor-activated signalling cascades. However, alterations in intracellular calcium, endothelin (ET), and vasodilators including nitric oxide (NO) and prostacylin are detrimental in the contribution to vasoconstriction and impaired vasodilatation, which can result in the onset of hypertension.

1.8.3 Endothelin

Endothelin-1 (ET-1) is a 21 amino acid peptide produced in the endothelium and is involved in cardiovascular diseases such as hypertension due to its potent vasoconstricting activities (Kirkby *et al.* 2008; Opitz and Ewert, 2006). By binding to its receptors, ET_A and ET_B, which belong to the G protein coupled receptor (GPCR) superfamily, it can possess long lasting effects on the vasculature such as vasoconstriction, production of reactive oxygen species (ROS) and inflammation of the vascular smooth muscle cells (VSMCs, Schneider *et al.* 2007) through activation of the ET_A receptor. In contrast to this, beneficial effects such as vasodilatation and NO production is mediated by ET_B receptors (Schneider *et al.* 2007).

1.8.4 Nitric Oxide

NO is a powerful vasodilator which plays an important cellular signalling role in both physiological and pathological processes. It contributes to vessel homeostasis through the inhibition of vessel constriction and smooth muscle cell growth/proliferation and platelet aggregation via inhibition of growth factors including platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF). In addition to this, NO inhibits leukocyte adhesion to the endothelium and expression of the antioxidant response genes (Kolluru *et al.* 2010; Moncada and Higgs, 2006). The endothelial layer of the blood vessel is known to be essential for the maintenance of vascular homeostasis via the

production of endothelial-derived nitric oxide (eNOS) and eNOS is responsible for most of the vascular produced NO. Impaired eNOS bioactivity is the major contributor to endothelial dysfunction, an important event in the pathogenesis of cardiovascular diseases including hypertension, atherosclerosis and diabetes. The mechanisms in defining the pathological process towards endothelial dysfunction and oxidative stress are still not fully understood. When functional, eNOS oxidises its substrate L-arginine (L-Arg) to L-citrulline and NO. This normal functioning requires the presence of the essential co-factor (6R)-5,6,7,8-tetrahydro-L-biopterin (BH_4) which is a potent reducing agent. During hypertension and vascular disease, the production of ROS in the vessel wall generates superoxide ($O_2^{\cdot -}$) which reacts with vascular NO to form peroxynitrite ($ONOO^-$). BH_4 is sensitive to oxidation by $ONOO^-$ and diminished levels of BH_4 promote $O_2\cdot^-$ production by eNOS, also referred to as “eNOS uncoupling” (Forstermann and Munzel, 2006, Figure 1.2). This loss in NO bioavailability can result in detrimental effects to the vessel wall such as induction of PGDF and VSMC proliferation which in time leads to remodelling of the vessel wall. Experimental inhibition of eNOS production by the vessel wall can be achieved by intervention with N(G)-Nitro-L-Arginine Methylo Ester (L-NAME) which has been successfully utilised as an experimental model of hypertension and will be discussed in more detail later on in this chapter.

1.8.5 The antioxidant defence system and oxidative stress

ROS are chemically reactive molecules and free radicals derived from molecular oxygen, a property which makes them highly reactive. These molecules are short lived and readily react with lipid bilayers and proteins (Winterbourn, 2008), and unless these radicals are terminated by antioxidants, this radical generation is exaggerated, thus generating oxidative stress and cell damage. Under physiological homeostasis and in the onset of oxidative stress, ROS levels are strictly modulated by the antioxidant defence system. This delicate system has evolved to protect the cells against the harmful properties of ROS, and to protect against the irreversible damage caused to the vasculature in the onset of oxidative stress. Low antioxidant bioavailability promotes cellular oxidative stress (Izzo *et al.* 2008). During the oxidative stress pathway oxidation occurs when an

electron is transferred from a substance to an oxidising agent to produce free radicals and cause cell damage, and in some cases cell death. Antioxidants are molecules which are capable of inhibiting the oxidation of other molecules. They are able to terminate the oxidative reaction chain by removing free radicals by oxidising themselves, therefore making them reducing agents. The antioxidant defence system is comprised of enzymatic ROS scavengers; including superoxide dismutase (SOD), catalase, glutathione peroxidase (GPX) and heme-oxygenase (HO-1) and non enzymatic ROS scavengers such as glutathione, urate and vitamins (Zadak *et al.* 2009). SOD catalyses the dismutation of $O_2^{\cdot -}$ into H_2O_2 and O_2. This H_2O_2 is then broken down further by catalase and GPX into H_2O and O_2. These ROS scavengers and their role in the development of Ang II induced hypertension are shown in Figure 1.2 and have been shown to be effective targets for treatment in hypertension, which will be discussed in more detail in Chapter 6.

Oxidative stress has a unique mechanism in many vascular diseases, and is primarily the result of imbalance between the generation of ROS and the antioxidant defence mechanisms. This results in an excess in oxidant levels produced in comparison to antioxidants in a state of injury, therefore there is a shift of the redox state towards an oxidizing state, and in turn oxidative damage occurs within the tissue in addition to DNA and protein damage (Davies, 2005; Kamata and Hirata, 1999). Vascular injury by oxidative stress most commonly occurs through the disruption of the NO signalling pathway which, as already mentioned, is known to protect the vessels and maintain homeostasis (Rodrigo *et al.* 2011). During this disruption, superoxide anions chemically react and inactivate NO's anti-inflammatory and vasodilatory functions. This leads to the formation of peroxynitrite, and uncoupling of eNOS (Figure 1.2). eNOS then undergoes transition in a change from being a vasoprotective enzyme, to become a superoxide-producing enzyme, therefore creating oxidative stress (Harrison *et al.* 2010). Oxidative stress is considered to be one of the key mechanisms responsible for the development of hypertension, with ROS playing an important role in homeostasis in the vascular wall (Paravicini *et al.* 2006). Sources of ROS in the endothelium include mitochondria, NADPH oxidase (NOX), xanthine oxidase and eNOS. NOX and mitochondria however are the key contributors towards cellular ROS production and has been implicated in various vascular diseases such as diabetes, pre-eclampsia and hypertension (Droge, 2002). The

mitochondrion is a key source of ROS formation and ATP generation, and is at the key core of cellular energy metabolism. Calcium (Ca^{2+}) is the key regulator of its function, and under physiological conditions, Ca^{2+} is beneficial for mitochondrial function. However dysregulation of mitochondrial Ca^{2+} homeostasis such as Ca^{2+} overload in response to stimuli such as Ang II can pose detrimental, and can lead to the enhanced generation of ROS, oxidative stress, apoptosis and DNA damage (Brookes *et al.* 2004). This increase in Ca^{2+} also results in reduced relaxation in the vessel wall (Izzo *et al.* 2008).

In the onset of hypertension, when hormones such as Ang II and the powerful vasoconstrictor, ET-1 are stimulated, there is a rapid increase of superoxide in the vessel wall, which is known to be primarily derived from NADPH. This cascade of events therefore leads to an increase in arterial pressure and vasoconstriction of the vessel wall through increased intracellular calcium concentrations (Lassegue and Griendling, 2004, see Figure 1.1). This research highlights the importance of oxidative stress and ROS in terms of having a prominent role in the onset of hypertension. When reviewing the research previously shown on animal models, it has been shown that knockout mice, which are deficient in ROS-generating enzymes such as NADPH oxidases, are less susceptible to high BP in comparison to their matched wildtypes (Landmesser *et al.* 2003). Cultured VSMCs from hypertensive patients have also displayed enhanced ROS production, and subsequent antioxidant reduction (Touyz *et al.* 2001). This research therefore implies ROS and oxidative molecules as an obvious target in terms of drug therapy.

As previously mentioned, one of the major source of ROS in the vessel wall is NADPH oxidase, particularly superoxide. The NADPH oxidases are a family of multi-subunit enzyme complexes known to generate both superoxide and hydrogen peroxide. Within the cardiovascular system, the major catalytic subunits are NOX1, gp91phox (NOX2), NOX3 and NOX4. These different isoforms share similar tertiary structures which include a six transmembrane domain containing four histidine residues which form two haem binding sites. There is then a long C-terminal cytosolic tail. They also require regulatory subunits in order to function, these being p22phox, p47phox, p67phox p40phox and RAC. All NOX isoforms require heterodimerization with p22phox as it is thought to be required for stabilisation of the protein expression (Rivera *et al.* 2010). However, whilst this

heterodimerization with p22phox is all that is needed for functional NOX4 activity, NOX1-3 requires association with all regulatory subunits (p22phox, p47phox, p67phox p40phox and RAC). For the purpose of this thesis, we will concentrate on NOX2 and NOX4, their structures are shown in Figure 1.3. There is strong evidence that these isoforms play a pivotal role in Ang II induced hypertension (Griendling *et al.* 2009), with global knockouts demonstrating reduced vascular injury in terms of decreased superoxide levels and increased NO bioavailability (Wang *et al.* 2001; Carlstrom *et al.* 2009; Touyz *et al.* 2005). All four subunits are upregulated in endothelial cells (ECs) and VSMCs in small vessels exposed to Ang II (Touyz *et al.* 2003). NADPH oxidase-derived ROS involves a cascade of events in which NADPH catalyses the reduction of molecular oxygen, which in turn functions as an electron donor and generates superoxide.

High levels of ROS, superoxide production via NADPH, and the subsequent loss of NO bioavailability and antioxidant genes contributes to the onset of vascular remodelling via VSMC proliferation (Zalba *et al.* 2000).These processes are associated with hypertension, because they contribute to the narrowing of the arterial lumen, and an increase in the vessel wall diameter, in addition to collagen formation which results in vessel stiffness (Rodrigo *et al.* 2003). These changes consequently result in increased peripheral resistance and blood pressure (Lacy *et al.* 2000). This remodelling by ROS occurs via the activation of several intracellular signalling cascades such as extracellular signal regulated kinases (ERKs), mitogen-activated protein kinases (MAPKs), protein tyrosine kinases and transcription factors, all of which have an influence in cell growth and vascular remodelling (Fortuno *et al.* 2005; Cai, 2005). ROS can also modulate vascular remodelling through the increase in extracellular matrix protein deposition. During the development of oxidative stress, macrophages and VSMCs secrete the enzymes metalloproteinases (MMPs) which promote degradation of basement membrane and elastin in the SMCs which causes damage and collagen build-up (Wainwright, 2004). Inflammatory cascades also contribute to the vascular remodelling in hypertension which is discussed further in section 1.8.6.

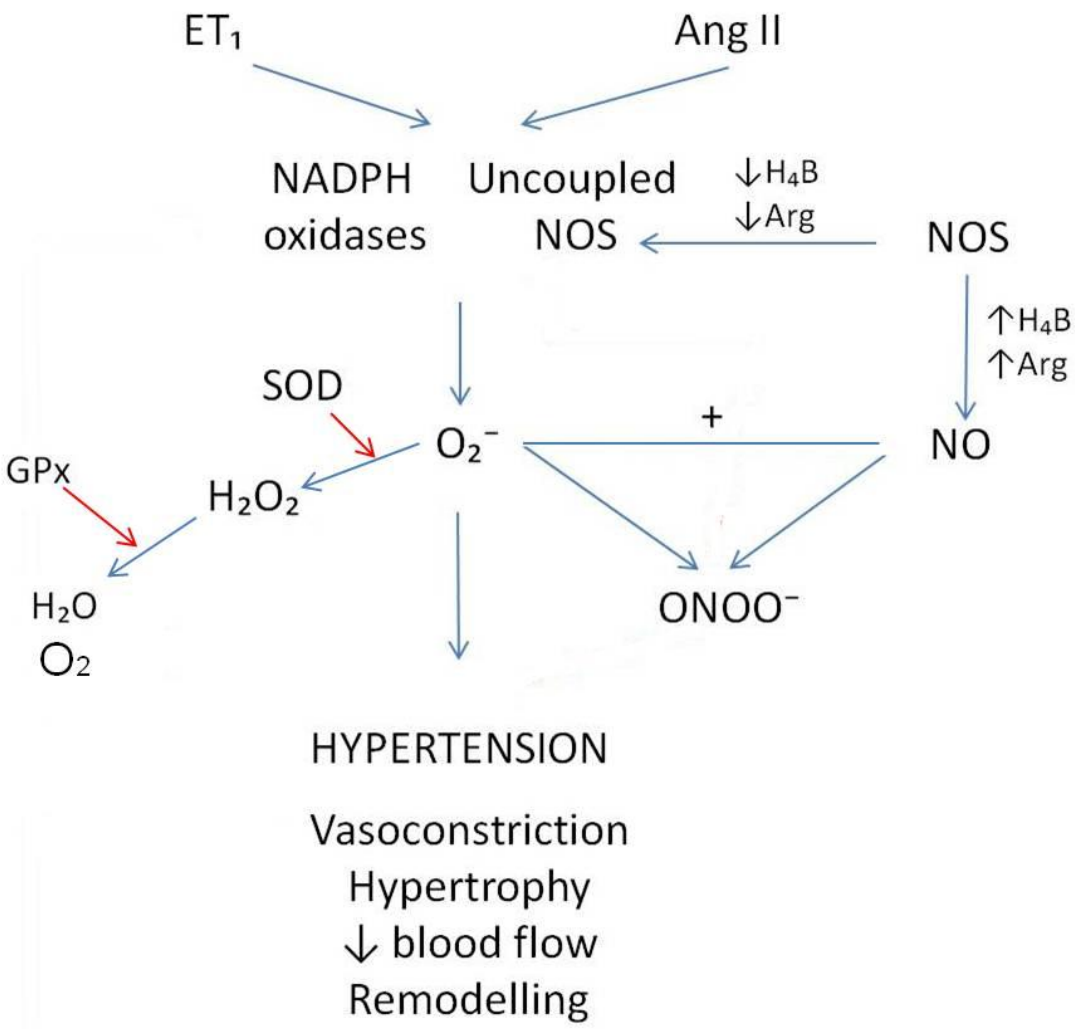


Figure 1.2. The pathways leading to hypertension via oxidative stress and ROS. Superoxide (O_2^-), hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$) are produced in excess as a result of an upregulation of hypertensive agents such as Ang II and ET-1. This upregulation causes superoxide production through NADPH and uncoupled nitric oxide synthase (NOS). NOS is uncoupled by a decrease in its substrate L-arginine or tetrahydrobiopterin (BH_4). In the presence of these substrates, NOS generates the antihypertensive NO to induce vasodilatation. During oxidative stress, NO reacts with superoxide to form peroxynitrite, which is also responsible for NOS uncoupling and inhibition of superoxide dismutase (SOD). SOD converts superoxide to hydrogen peroxide (reaction illustrated in red) which can further be degraded to water and oxygen which is cleaved by the antioxidant glutathione peroxidase (GPX, reaction shown in red). This cascade of events leads to the imbalance of the redox state and a shift towards the oxidising state which in turn results in hypertension and vascular damage. Adapted based on a figure by Rodrigo *et al.* 2011.

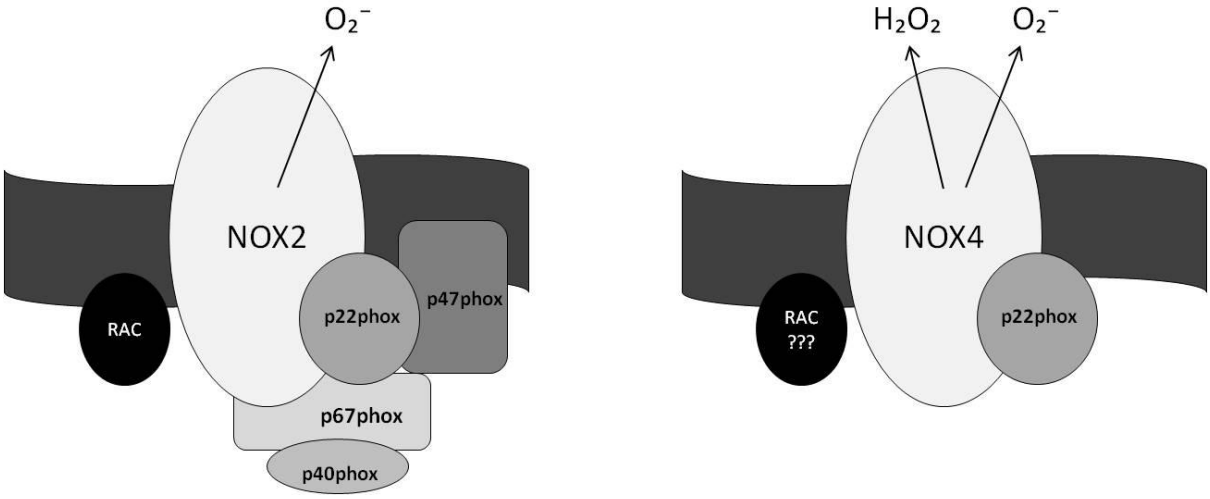


Figure 1.3. Model of NADPH oxidases 2 and 4 and their regulatory subunits. Both NOX 2 and NOX4 require p22phox for activity, however NOX 2 requires additional regulatory subunits (p47phox, p67phox, p40phox and RAC). NOX2 predominantly generates superoxide on activation, however NOX4 is thought to generate hydrogen peroxide also. Based on a figure by Munzel *et al.* 2010.

1.8.6 The role of classic inflammation in Ang II induced hypertension

During the onset of cardiovascular disease and vascular injury endothelial dysfunction occurs, which results in the imbalance between the vasodilating and vasoconstricting substances produced by the endothelium. This imbalance has been implicated in the pathophysiology of hypertension due to the reduced vasodilatation and the pro-inflammatory state of the disease. These initial steps proceed to vascular inflammation, and in more serious cases atherosclerosis, which is thought to be mediated at least in part by ROS (Libby *et al.* 2001). ROS induces vascular remodelling and inflammation which leads to recruitment of pro-inflammatory mediators and adhesion molecules such as vascular cell adhesion molecule (VCAM-1) on the endothelium, and proliferation of VSMCs (Drummond *et al.* 2011). This pathway also causes up-regulation of pro-inflammatory cytokines such as the interleukins e.g. IL-1, 6 and 12. The inflamed site on a vessel wall is readily made up of lipid, monocytes and T lymphocytes (Brasier *et al.* 2002). These cell types are recruited to the sub-endothelial space at the onset of injury through the action of chemotactic cytokines and adhesion molecules expressed on the surface of the endothelium. This continuous recruitment of cells on the endothelium then migrate into the vessel wall and monocytes differentiate into macrophages (Figure 1.4) which results in the increased production of pro-inflammatory cytokines and chemokines, hence inflammation and remodelling occurs. Experimental models of atherosclerosis have also linked the RAS with a role in atherogenesis and vascular inflammation via NADPH oxidases, with ACE inhibitors targeting this system and reducing end point vascular inflammation by up to 70% over 12 weeks (Hayek *et al.* 1999). In a rat Ang II renal model, blockade of Ang II by ACE inhibitors decreases infiltration of inflammatory cells and growth factors (Mezzano *et al.* 2001). It was also shown that ARB administration reversed endothelial dysfunction and reduced the constriction of resistance vessels by promoting endothelial relaxation (Schiffrin *et al.* 2000). Ang II upregulates vascular cell adhesion molecule (VCAM-1), monocyte chemotactic protein-1 (MCP-1) and intracellular adhesion molecule (ICAM-1) through ROS production. These important adhesion molecules bind onto the surface of the endothelium before being recruited into the vessel wall to promote VSMC proliferation. Studies have shown that by blocking the RAS pathway with ACE

inhibitors and ARBs, expression of these adhesion molecules are significantly reduced (Navalkar *et al.* 2001; Dol *et al.* 2001). It has also been shown in humans that treatment with ARBs reverses endothelial dysfunction in large arteries (Prasad *et al.* 2000). Upregulation of the Ang II pathway is therefore known to cause endothelial dysfunction through the inhibition of endothelial NOS production which then triggers the inflammatory cascade of events leading to vascular inflammation. The mechanism by which Ang II is thought to play a part in the vascular inflammation cascade is thought to be due to the Ang II signal transduction mechanisms overlapping the typical pro-inflammatory cytokine pathways. Ang II can indeed stimulate the NF-κB pathway which induces the inflammatory cascades. Inhibition of the NF-κB pathway is a therapeutic target in preventing inflammation as it was shown to block Ang II induced production of IL-6, VCAM-1 and MCP-1 expression (Tummala *et al.* 1999; Hernandez-Presa *et al.* 1997; Han *et al.* 1999). ACE inhibitors and ARBs however inhibit this action, making the RAS pathway an additional target for anti-atherosclerotic therapies in addition to targeting ROS and NADPH oxidases alone (Brasier *et al.* 2002)

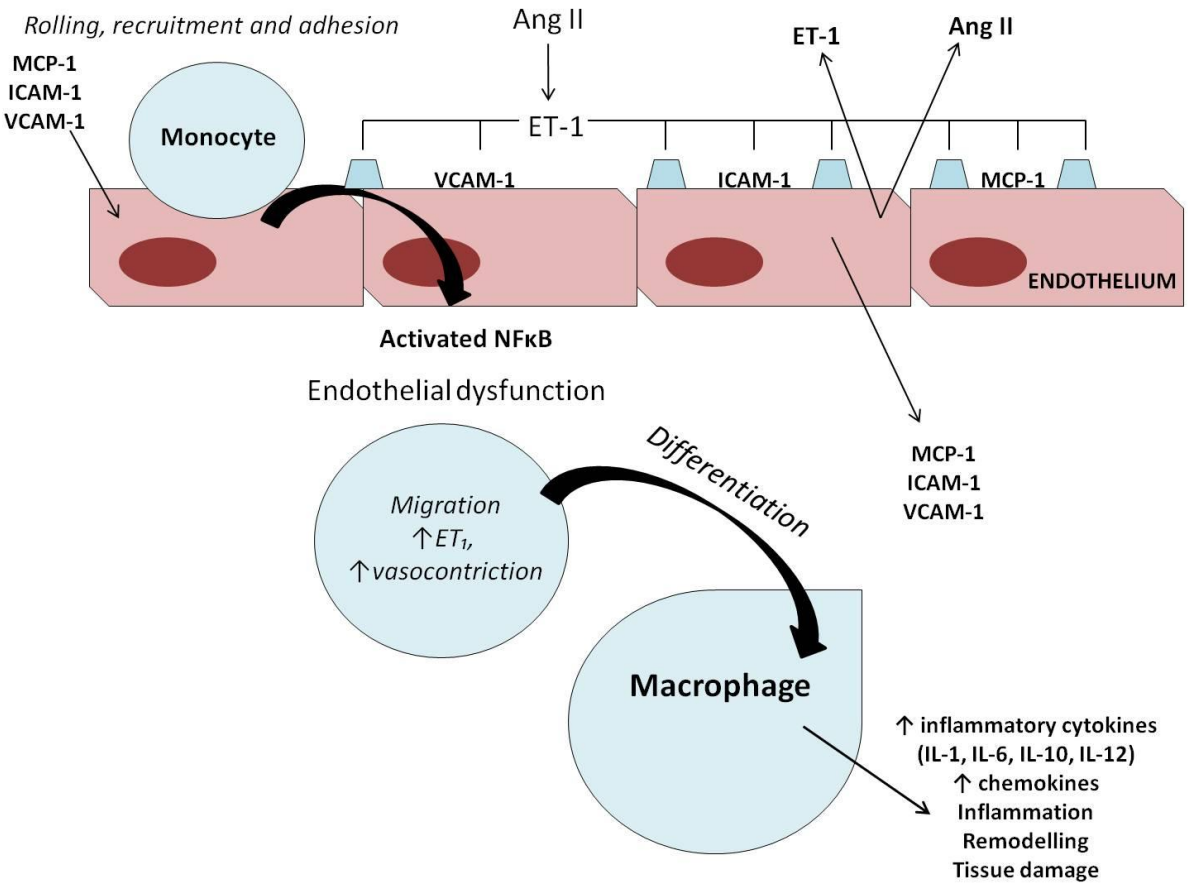


Figure 1.4. Schematic diagram illustrating the mechanism by which Ang II promotes vascular inflammation. Ang II is activated through the RAS as illustrated previously, which then activates the endothelin (ET) pathway and stimulates ET receptors on the endothelium which results in vasoconstriction and induces endothelial dysfunction. Ang II produced in the endothelium then induces monocyte recruitment and adhesion molecules (VCAM-1, MCP-1, ICAM-1) onto the endothelium and progress into the VSMCs via activated NFκB. This migration of the monocytes and upregulation of Ang II and ET-1 induces vasoconstriction. The cells then differentiate in the VSMC into macrophages in which production of pro-inflammatory cytokines and chemokines occur and a progressive inflammatory state develops within the vessel wall.

1.9 Experimental models of hypertension

Animal models are routinely used in order to understand the pathogenesis of a disease and develop treatment. Various models of experimental hypertension have been developed to gather information on the pathophysiology of hypertension. These models are also used in the pharmacological screening of potential antihypertensive agents. The animal models of hypertension which are described here share many features which are found in human hypertension. They have been developed to utilize the etiological factors which are responsible for human hypertension such as excessive salt intake, hyperactivity of the renin angiotensin aldosterone system (RAS), and genetic factors. The various types of animal models of hypertension being used to date include: renovascular, dietary, endocrine, neurogenic, psychogenic and genetic hypertension, (Badyal *et al.* 2003). There now follows a description of some of the more commonly used models, before extending the introduction to calcitonin gene-related peptide (CGRP), the knowledge of which has already been gained using some of these models.

1.9.1 Angiotensin II as an experimental model of essential hypertension

Angiotensin II (Ang II) induced hypertension is one of the most common hypertensive models used to date and has been studied as a hypertensive model since the agonist was available in pure form. There are many reasons behind this, one of them being that plasma renin activity is significantly increased in renovascular hypertension and in around 16% of patients with essential hypertension. It is known that Ang II differs from other vasoconstrictors in that its pressor effect is self-potentiating and cumulative. Infusion of subpressor doses of Ang II is known to ultimately lead to hypertension. Gradual development of hypertension induced by long term administration of these doses of Ang II is said to mimic the human high renin (Ang II) hypertension, (Li and Wang, 2005). However the mechanisms for this hypertension development still remain unclear.

The Ang II infusion model has been well established within our laboratory (Liang *et al.* 2009) in which an osmotic mini-pump is implanted under the skin in the

scapular region and delivers a constant dose of Ang II. There are a range of doses of Ang II used by several research groups, ranging from 0.3-1.44 mg/kg/day (Qin, 2008). These reported doses were administered over a period of between 7 to 28 days, depending on how severe the model was and the outcomes required. Studies looking at vascular remodelling and aortic stiffness reportedly use a dose of 1.4mg/kg/day for a 30 day period (Cunha *et al.* 2006; Yamazaki *et al.* 2011) whilst other groups use subpressor doses of 0.5mg/kg/day for 2 months when looking at models of diabetic pulmonary fibrosis (Yang *et al.* 2011). It is widely reported that a significant increase in BP is observed in the Ang II infused subjects between days 7 to 9 compared to their vehicle controls (Widder *et al.* 2009). This increase has also been reported previously in studies within our group, in addition to the onset of vascular inflammation (Liang *et al.* 2009; Bodkin *et al.* 2011, unpublished). During the continuous Ang II infusion, there is an elevation of intrarenal Ang II via the AT_1 receptor and an increase in ET-1 production. This results in a reduction in renal function and NO/eNOS bioavailability therefore hypertension occurs. This model is therefore also associated with a predominant increase in oxidative stress and vascular inflammation/remodelling as shown previously (Yang *et al.* 2011; Liang *et al.* 2009). The main advantage of this model over others is that Ang II infusion results in significantly elevated BP and vascular inflammation over a relatively short period of time (Widder *et al.* 2008) with reliable results.

1.9.2 Other models of essential hypertension

Genetic hypertension- The spontaneously hypertensive rat (SHR)

In 1963, Okamoto and Aoki developed a new model of experimental hypertension. This model was unique as it required no physiological, pharmacological or surgical intervention. They developed the spontaneous hypertensive rat (SHR) by meticulous genetic inbreeding which resulted in 100% of the progeny having naturally occurring hypertension, (Okamoto and Aoki, 1963). This is known as an excellent model of hypertension which mimics human clinical essential hypertension. Like human beings, the onset of hypertension occurs with

advancing age (Pinto *et al.* 1998). Hypertension is observed in these models as early as 12 weeks of age, (Trippodo and Frohlich, 1981). And by 40 weeks, SHR develop characteristics of cardiovascular disease such as hypertrophy and vascular remodelling (*Conrad et al.* 1995). These models can be used not only in the pathogenesis and therapy, but also prophylaxis in essential hypertension and its relative complications, (Escudero *et al.* 2008). In a letter by Kundou and Rao, it was stated that a literature search shows that with a high number of publications, the SHR is the most studied model of hypertension at present (Kundou and Rao, 2008). However there is one major downfall regarding this model; this being the rat and not a mouse. We can currently develop genetically modified mice, these being gene knockouts and transgenic. This has not been fully established in rats at present, with only a very limited number of genes being able to be knocked out in the rat. Therefore it serves to be a problem in looking at the effects of gene deletion in this model.

L-NAME

Chronic inhibition of NO synthase by L-NAME is a well established model of experimental hypertension. L-NAME vascular effects have been demonstrated to inhibit the vasodilatory effects of NO by inhibiting eNOS activity. This inhibition of NO leads to the subsequent production of ROS which induces the hypertensive response and increase in arterial pressure. There is also evidence for the contribution of the RAS and the production of superoxide anions in the induced elevation of BP. Therefore, L-NAME induced hypertension allows the detection of the effects of several classes of antihypertensive drugs in rodent. One of the suggested methods of administering L-NAME is giving it in drinking water. The reported effective doses are 1mg/ml over 6 days (Cunha *et al.* 1993), 1g/L over 8 weeks (Kanematsu *et al.* 2008), 100mg/kg/day for 21 days (Laflame *et al.* 1998) and 60mg/kg/day for 7 days (Rodriguez *et al.* 1998). L-NAME has also been shown to work effectively when injected intravenously (30mg/kg), with acute responses (De Angelis *et al.* 2006).

1.9.3 Experimental models of secondary hypertension

Renovascular hypertension

Renovascular hypertension is very common model of hypertension used and the RAS plays an important role, (Ganong. 1999). Experimentally, renal hypertension is initiated by renal artery constriction via tubule clipping or total removal of a kidney, which in turn activates the RAS and sympathetic nervous system, (Mok *et al.* 1985). The decreased blood volume leads to sympathetic stimulation. Renin is secreted in the kidney when this sympathetic activity is increased. This in turn converts angiotensinogen to Ang I, which is then converted to Ang II by ACE. The vasoconstricting properties of Ang II cause the rise in BP and release of aldosterone. This causes salt and water retention which ultimately leads to increased blood volume and hypertension, (Guyton, 1998).

Dietary Hypertension

Dietary hypertension has been studied in animals by increasing salt intake, or more recently, a high fat diet. With regard to an increase in salt, hypertension was induced in rats by replacing drinking water with 1-2% sodium chloride for 9-12 months, (Boura *et al*. 1964).This chronic ingestion of excess salt was said to produce hypertension which mimics human hypertension morphologically, (Dahl *et al.* 1960).

More recently, a high fat diet has been used as a model of hypertension and is established within our laboratory (Marshall *et al.* 2009). A 4-35% high fat diet in mice for 12- 40 weeks is said to mimic the high fat diet consumed by humans of the Western world and leads to obesity induced hypertension (Friedman and Halaas, 1998; Reimer and Ahren, 2002). Animals display moderate increases in BP, severe visceral obesity, increased adipose tissue mass and increased blood glucose levels. End organ damage is also shown to be severe with increased vascular hypertrophy, (Motter and Ahern, 2008; Chengyi *et al.* 2004).

Endocrine hypertension

Mineralocorticoids cause sodium and water retention until diuresis occurs due to pressure on the kidneys. It was first demonstrated by Selye *et al.* (1957) that deoxycorticosterone salt (DOCA) causes hypertension in the rat. There is increased DOCA-induced reabsorption of salt and water leading to increased blood volume, and therefore an increase in BP, (Selye *et al*. 1957). In this model, there is also increased vasopressin secretion which inevitably leads to water retention and vasoconstriction. Altered activity of the RAS leads to increased sympathetic activity, (Hakim and Goyal, 2000). Other Mineralocorticoids, such as aldosterone, and glucocorticoids can produce this hypertension as first shown by Knowlton *et al.* (1952).

This method of hypertension (DOCA) is dependent on salt since neither administration of DOCA or removal of renal mass is effective in increasing the BP alone. To produce hypertension, animals (mainly rats) are given a diet high in sodium chloride (NaCl) by replacing drinking water with 2% NaCl solution, alongside the administration of DOCA, (Manhiani *et al.* 2009). DOCA salt and Ang II are often combined to create a more severe model of hypertension than DOCA or Ang II individually (Kirchoff *et al.* 2008; Weiss and Taylor, 2008).

Another method of endocrine hypertension is adrenal regeneration hypertension. This is performed by unilateral nephrectomy followed by removal of the right adrenal gland and enucleation of the left adrenal gland. Drinking water is then replaced by saline. Hypertension is induced within 2 weeks. This model is used mainly to study the roles of steroids in the development of hypertension, (Grisk *et al.*1999).

1.10 BP measurement in murine studies

At present, there are two techniques commonly used to measure BP (BP) in conscious rodents, these being tail cuff plethysmography and radiotelemetry. Within our group, tail cuff plethysmography has been developed and used successfully on numerous occasions, (Clark *et al.* 2007; Liang *et al.* 2009). However in recent years, developments in the use of radiotelemetry have made us consider whether the tail cuff method is still as accurate and precise.

Radiotelemetry is the “gold standard” and many research groups are now using this measurement as their preferred method due to reviewers comments whilst trying to publish their research. The main reason for the preferred use of radiotelemetry over tail-cuff is primarily due to the animal not being restrained, which is the major cause of artefact reading obtained through tail-cuff due to stress. The American Heart Association have also concluded that when referring to the available tail cuff method on the market, some are proven to be more accurate than others, (Feng *et al.* 2008). Comparative studies have been carried out using parallel groups of mice and it has also been shown previously within our group that when mice are trained correctly, there are no variations in the BP measurements observed from tail-cuff plethysmography compared to radiotelemetry (Marshall *et al.* 2009).

Tail cuff plethysmography uses volume pressure recording to measure BP in restrained and conscious mice. The technique involves restraining the animal in a humane clear plastic restraint with a fitted open nose cone to allow breathing, and then placing an occlusion cuff at the base of the tail which occludes the blood flow, and a volume pressure recording cuff towards the tip of the tail, which measures the change of pressure resulting in the returning blood flow. As the blood returns to the tail, it swells. This is measured as systolic pressure. Diastolic pressure is calculated when the tail ceases swelling. Both measurements can then be used to calculate MAP.

In order to reduce stress and artificial readings, mice undergo at least 14 days of BP training before readings are recorded. This allows the animals to get used to the experimental conditions and restraints, which cause them to be more relaxed and hence a more accurate reading is obtained. When measurements are being recorded, the first five measurements are discarded and known as acclimatisation measurements in which the mouse is still acclimatising to the environment. In order to achieve accurate and precise recordings, BP should be measured at an ambient room temperature of 27°C in order for the blood vessels in the tail to be relaxed and dilated, allowing blood flow changes to be reliably observed, (Whitesall *et al.* 2004; Feng *et al.* 2008).

Radiotelemetry involves the implantation of a fluid filled catheter into the aortic arch of the mouse. The catheter is then attached to a battery operated transmitter

unit, usually implanted underneath the skin of the mouse. This allows the transmission of data to a receiver pad normally located under the cage. The method of implantation is known to vary between different research groups, however the implantation into the aortic arch via the carotid artery is the most widely used in recent publications (Kramer and Kinter 2003; Samuelsson *et al.* 2008). Mice are then allowed time to recover from the surgery before measurements commence. Both experimental techniques have several advantages and disadvantages;

Advantages and disadvantages of tail cuff plethysmography and radiotelemetry

One of the major advantages of the tail cuff BP recording system is the cost. After the initial payment for equipment, very little ongoing costs or consumables are required, and those that are e.g. replacement air bladders for the inflatable cuffs are inexpensive. However, in terms of radiotelemetry, a much larger initial outlay for equipment and data analysis facilities are required. There is a huge amount of ongoing maintenance required for the fluid filled catheters themselves, as each catheter has to be reconditioned and must be replaced every 4-5 uses (Kramer and Kinter 2003). Comparative studies have suggested that of the two main types of tail cuff measurements being used to date (pulse based and blood flow based), pulse based measurements are more accurate than blood flow based, which show more variation from comparative results obtained with radiotelemetry (Feng *et al.* 2008). However, in general, there is acceptable agreement between the measurement of mouse pressures by tail cuff and radiotelemetry when used under the correct conditions (Whitesall *et al.* 2004, Marshall *et al.* 2009, unpublished).

Tail cuff techniques have previously been shown to be inaccurate at both low (<50mmHg) and high (>180mmHg) measurements. This suggests that this may not be a suitable measurement of BP in more severe models of hypertension (Feng *et al.* 2008). Some studies have also shown that measurements obtained from tail cuff readings are subjected to a higher degree of variation in comparisons to radiotelemetry readings. One such study even concluded that the tail cuff technique was only suitable in models where a BP change of ≥ 15-20mmHg could be anticipated to account for variation between measurements

(Gross and Luft 2003). This was also confirmed by Whitesall *et al.* (2004), who added that when the experimental design was more suited to tail cuff measurements (e.g. if BP recordings are required for a longer period of time than the lifetime of a telemetry transmitter battery), then tail cuff plethysmography was a valuable technique (Whitesall *et al.* 2004).The measurements obtained through the use of tail cuff demonstrate a larger degree of variation in comparison to telemetry. It is also subject to a higher incident of artifactual measurements e.g. an artifactual increase in BP resulting from a stressed mouse (Kramer and Kinter 2003). Therefore, it is extremely important that the mice stay relaxed in order for an accurate measurement to be obtained using tail cuff. As such, considerable supervision and interaction is required from the person in charge of the experiment to ensure that the cuffs do not slip off the tail due to the mouse moving within the restraint, and that the animal is not stressed (Kramer and Kinter 2003).

As already mentioned, it has been said that radiotelemetry is in fact todays “Gold standard” when measuring BP, and has some advantages over tail-cuff plethysmography which we used for these studies. However, a comparative studies result in similar recordings in both methods. This therefore suggests that tail-cuff is still a valuable and accurate tool when measuring BP. Both methods are therefore known to provide reliable and accurate results. However, there are still some advantages which radiotelemetry has over tail-cuff (e.g. no need to restrain the animal, can record 24hr/day) which makes it a more suitable method of recording BP in certain experimental models. Tail-cuff plethysmography was the chosen method for recording BP in this thesis.

1.11 Sensory nerves and calcitonin gene-related peptide (CGRP)

"Neurogenic inflammation" is the term commonly used to describe a series of inflammatory responses created by the release of neuropeptides from terminals of primary sensory neurones in response to injury (Maggi, 1991; Holzer, 1992; Geppetti and Holzer, 1996; Fernandes *et al.* 2009). These sensory neurones are abundant in almost all tissues at the perivascular level (Holzer, 1992). Released neuropeptides include the vasodilator calcitonin gene-related peptide (CGRP), in addition to others including substance P (SP) and neurokinin A (NKA, Brain and Cox, 2006). These neuropeptide-containing neurones are also known as polymodal nociceptors due to their ability to be activated by chemical, thermal and mechanical stimuli (Maggi, 1990). Studies involving the mechanisms behind pain and neurogenic inflammation have shown that they are very sensitive to the action of capsaicin (pungent chilli pepper extract, Maggi and Meli, 1988), which acts as a selective agonist of the V1 subtype of the transient receptor potential (TRPV1) family of cation channels (Brain and Cox, 2006). Activation of the receptor induces the opening of a central pore which is permeable to the influx of extracellular cations and calcium. A rise in intracellular calcium leads to depolarization of the neuronal membrane which in turn causes the subsequent release of sensory neuropeptides, including CGRP. Capsaicin is a potent compound, regularly used as a research tool to investigate the role of sensory nerves. Topical application of capsaicin to the murine ear results in increased blood flow, mediated by CGRP and substance P receptors (Grant *et al.* 2002). Continuous application of this agonist however leads to the depletion of sensory nerves in which prolonged administration results in blockage of conduction in C fibres and a loss of chemical sensitivity due to impairment of signal transduction at the afferent nerve terminal (Dray and Dickenson, 1993). By using capsaicin sensory nerve degeneration, it has been possible to study the role of sensory nerves in BP regulation (Burg *et al.* 1994; Manzini and Bacciarelli, 1988; Wang, 2005).

CGRP is derived from the calcitonin gene and exists in two forms, named alpha CGRP (αCGRP) and beta CGRP (βCGRP). Whilst these two forms share the same biological activities, they are formed from two distinct genes at different sites on chromosome 11 in the human. CALC I forms calcitonin and αCGRP whereas CALC II forms βCGRP, (Alevizaki *et al.* 1986). αCGRP synthesis is caused by alternative splicing of the calcitonin gene (Hay, 2007; Kittur *et al.*

1985). βCGRP is known to be transcribed from its own distinct gene (Steenbergh *et al.* 1986). These two homologues share >90% homology and differ by only three amino acids in the human (Morris *et al.* 1984; Amara *et al.* 1985; Steenbergh *et al.*1986, see figure 1.5). They also share similar biological properties (Wimalawansa *et al.* 1990).

Human αCGRP has four domains (Conner *et al.* 2002). The N-terminus domain is made up of the first seven residues which have a ring like structure, held together by a disulphide bridge. Removal of this N-terminus converts CGRP into its antagonist $CGRP_{8–37}$. The second domain is an α helix lying between residues 8–18. Deletion of this domain is known to cause a 50–100 fold decrease in affinity (Rovero *et al.* 1992). Within this, R11 and R18, located on the hydrophilic face of the α helix play a vital role in promoting high affinity binding (Howitt *et al*. 2003). The next domain at residues 19–27 consists of a β or γ twist (Conner *et al.* 2002). The C-terminus lies at residues 28–37, and contains two turn regions which form a putative binding epitope (Carpenter *et al.* 2001; Breeze *et al.* 1991, see figure 1.6 for 3D structure of CGRP 1-19). Comparison of this to the βCGRP structure indicates that each form of the peptide is virtually identical (Morris *et al.* 1984; Bell and McDermott, 1996).

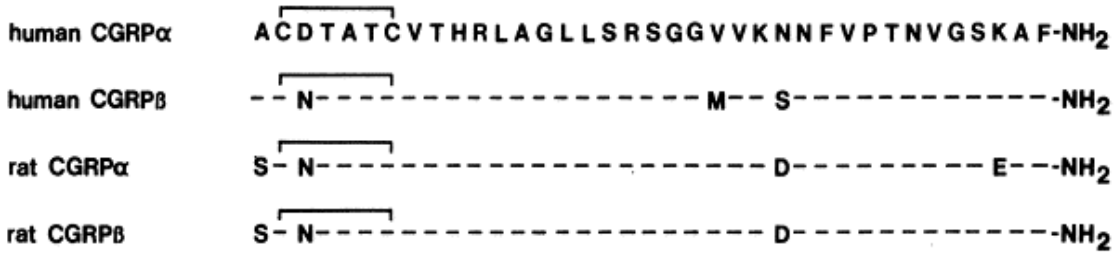


Figure 1.5. Amino acid sequence of human and rat α and β CGRP. Peptide amino acid sequences of α and β CGRP isoforms showing that the two isoforms differ by only 3 amino acids and therefore share >90% homology. Figure taken and adapted from Van Rossum et al. 1997.

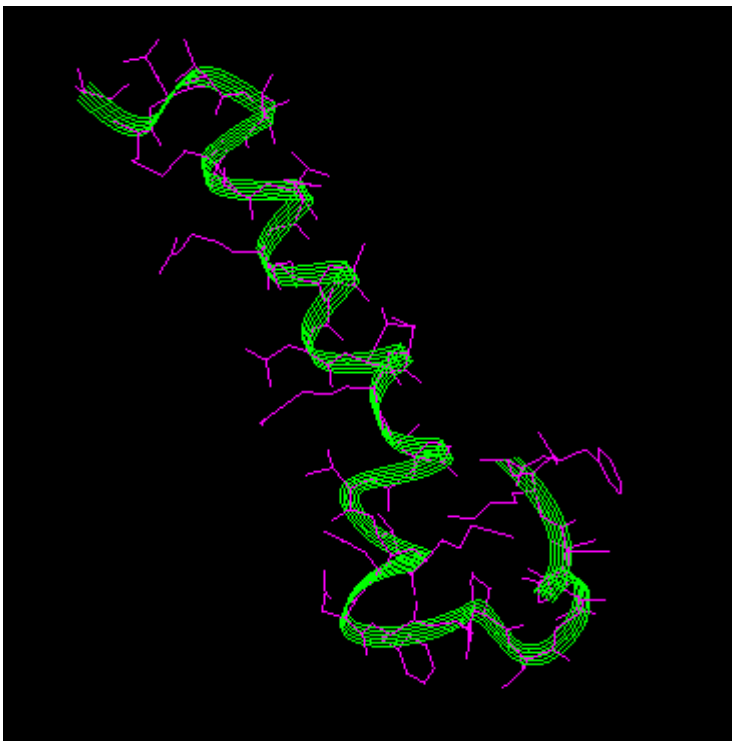


Figure 1.6. Three dimensional structure of CGRP 1-19 (taken from Breeze *et al.* 1991).

1.12 Synthesis and localisation

CGRP is known to be derived from the tissue-specific splicing of the primary transcript of the calcitonin (CT)/CGRP gene. The importance of CGRP as a neuropeptide was discovered in 1982 through immunohistochemical research in the thyroid of aging rats and medullary thyroid carcinomas in man, which were found to contain an alternative peptide product from the calcitonin gene (Amara *et al.* 1982). The alternative peptide, CGRP, is produced by tissue-specific alternative splicing of the calcitonin messenger ribonucleic acid (mRNA) transcript. In the thyroid the dominant product is calcitonin, whereas CGRP is predominantly expressed throughout the central and peripheral nervous system. If exon 4 is expressed in the mature protein, it forms calcitonin, whereas expression of exon 5 and 6 leads to production of CGRP. However, the mechanism that governs the alternate splicing remains unclear. CGRP mRNA is translated into a 121 amino acid pro-hormone, which is then cleaved to form the mature 37 amino acid peptide.

CGRP and its receptors are distributed widely throughout the central (brain and spinal cord) and peripheral nervous systems, (Gibson *et al.* 1984; Lundberg *et al.* 1985; Brain and Grant, 2004). In the periphery, it is primarily found within sensory neurons, these being many small, unmyelinated C-fibres, and larger myelinated Aδ-fibres. These fibres are known to also contain other peptides, the most common being the tachykinins, especially SP and NKA, in addition to non-peptide neurotransmitters such as glutamate, (Lundberg *et al.* 1985).

The majority of CGRP within the body was suggested to be αCGRP and primarily expressed in neurons of the peripheral and central nervous system (Schutz *et al.* 2004). βCGRP was thought to be primarily expressed in the gut, including sites such as the enteric nerves (Mulderry *et al.* 1985, 1988) and pituitary gland (Petermann *et al.* 1987; Jonas *et al.* 1985), however studies have reported this isoform to be a potent vasodilator in the coronary vasculature of rats, and also expressed in human umbilical vein endothelial cells (HUVEC) therefore suggesting this isoform to also have a role in these tissues (Holmann et al. 1986; Luo *et al.* 2008).

Several studies have documented on the localisation of CGRP in the vasculature as described previously in a review by Bell and McDermott (1996). CGRP-

containing fibres have been found to be closely associated with blood vessels, therefore ensuring that CGRP release is localised perfectly for its role as a vasodilator (Holzer, 1992; Bell and McDermott, 1994). These CGRP-containing fibres have been shown to innervate the heart (Wimalawansa, 1996). Localisation of CGRP nerve fibres have also been detected in the ventricles of pigs (Miyauchi *et al.* 1988), rats (Mulderry *et al.* 1985) and the guinea pig (Ishikawa *et al.* 1987). However it was shown that higher amounts of CGRP-immunoreactivity is found in the atria compared to the ventricles (Mulderry *et al.* 1985; Lundberg *et al.* 1985; Ishikawa *et al.* 1987).

Autoradiographic and histological methods have localised CGRP in coronary arteries, veins, heart valves, ventricles, atria, coronary arterioles and endocardium in the rat (Sigrist *et al.* 1985; Mulderry *et al.* 1985), humans and guinea pigs (Coupe *et al.* 1990; Rechardt *et al*. 1986; Opgaard *et al.* 1995). However it is proposed that CGRP immunoreactivity is at a lower density in these coronary tissues compared to the major blood vessels (Lundberg *et al.* 1985).

McCulloch and co workers identified a high density of CGRP immunoreactive fibres in the abdominal aorta and pial arterioles of the rat (McCulloch *et al.* 1986). In addition to this, binding sites with high affinity for CGRP and CGRP-immunoreactive fibres have been detected in the surrounding nervous and connective tissue of the myocardium and coronary vessels in the rat (Yoshizaki *et al.* 1987) and pig (Miyauchi *et al*. 1988). When looking more closely, these CGRP fibres are observed at the junction of the adventitial layer and pass through into the muscle layer of the vessel (Holzer, 1988). In addition to the larger vessels of the coronary vasculature, these fibres have also been located in the smaller arteries of the respiratory, gastrointestinal and even dermal vasculature (Holzer and Guth, 1991; Dalsgaard *et al.* 1989).

Non-neuronal sources of CGRP have also been suggested more recently. A study by Wang *et al.* showed that human lymphocytes constitutively express both αCGRP and βCGRP (Wang *et al.* 2002). Interleukin-1β has also been shown to induce βCGRP in human type ii alveolar epithelial cells (Li *et al.* 2004). More recently, in a study by Hou *et al.* (2011) CGRP was widely detected in epidermal keratinocytes of the human skin with post herpetic neuralgia, with βCGRP being the predominant isoform expressed. These cell types were also found to express

the CGRP receptor components, calcitonin-like receptor (CLR) and receptor activity modifying protein 1 (RAMP1). It is thought from this study that CGRP expressed in the keratinocytes may modulate epidermal homeostasis and contribute to chronic pain during injury and inflammatory conditions (Hou *et al.* 2011). Another non-neuronal source of CGRP, which of great interest at present, is the circulating endothelial progenitor cells (EPCs) which have a role in the maintenance of endothelial homeostasis and vascular repair. Abnormalities and EPC senescence upregulation is associated with the onset of hypertension (Van *et al.* 2006). Ang II has also been shown to accelerate this senescence through the induction of oxidative stress (Imanishi *et al.* 2005). CGRP has been shown recently to inhibit this Ang II induced endothelial progenitor cell senescence through upregulation of Klotho expression (Zhou *et al.* 2010). In addition to this, studies have shown CGRP and its receptor components expression in cultured rat vascular smooth muscle cells and bovine endothelial cells, whereby CGRP administration to these cells results in vasorelaxation via a cyclic adenosine monophosphate (cAMP)-dependent and/or endothelium-dependent mechanism (Kato *et al.* 1995; Hirata *et al.* 1988).

1.13 Adrenomedullin and Intermedin

Adrenomedullin (AM) is a 52 amino acid vasodilator peptide discovered in 1993. As a regulatory peptide, it also belongs to the calcitonin family of peptides, sharing its sequence homology with CGRP (Bell and McDermott, 2008). It is expressed in vascular walls and produced by tissues such as the adrenal medulla, lung, kidney, neurons, vascular endothelium, VSMCs, adventitial fibroblasts and the heart (Jiang *et al.* 2004). It is known to play an important role in the regulation of circulation, as bolus administration of adrenomedullin into the circulation results in a hypotensive effect (Kitamura *et al.* 1993; Ishiyama *et al.* 1995). It also plays a role in cardiac stretch and dilation of coronary arteries through inhibition of oxidative stress and endothelial cell apoptosis (Kato *et al.* 2005). Patients with enhanced CVD display elevated AM levels in the plasma. In terms of mechanistic action, when AM is administered to subjects, AM causes dilation of the blood vessels via both endothelium dependent and independent pathways, similar to CGRP's mode of action (Hayakawa *et al.* 1999; Nishimatsu *et al.* 2001; Kato *et al.* 2005). Studies with cultured VSMCs indicate a protective mode of action for AM,

as it is shown to inhibit vascular damage (Kato *et al.* 1997) and proliferation (Liang *et al.* 2009). This result is also mirrored in rodent models where administration of AM prevents the advancement of vascular remodelling over a prolonged period of time (Tsuruda *et al.* 2005). AM has also been shown to suppress the vascular inflammation and hypertensive response of rats which were chronically infused with Ang II (Tsuruda *et al.* 2005). Also, *in vitro* studies showed that the generation of ROS produced in SMCs, after treatment with Ang II, was inhibited by AM (Yoshimoto *et al.* 2004).

In order to study the functions of AM, genetic animal models have been developed. It has been shown that AM is important in embryonic development using AM gene-targeted null transgenic (TG) mice (AM+/-) from two different research groups (Shindo *et al.* 2001; Shimosawa *et al.* 2002). AM-/- mice resulted in a fatal phenotype with the presence of hydrops fetalis, thin vascular walls and smaller hearts. This was also confirmed by Dackor *et al.* (2006), using TG mice (Calcrl-/-) with a targeted deletion of the gene that encodes the CL receptor which is an identified component for the AM receptor. These Calcrl-/- embryos validated the previously described AM-/- mice. Due to the AM-/- mice being embryonically lethal, many laboratories have focused on the AM+/- mice as a model of compromised AM gene expression. AM+/- and AM overexpressing TG mice have been used to demonstrate the protective function of AM in atherosclerosis (Imai *et al.* 2002) and Ang II infusion (Niu *et al.* 2003; Niu *et al.* 2004). It was shown by these groups that AM+/- mice had an increased degree of cardiovascular hypertrophy and fibrosis and renal dysfunction with glomerular sclerosis after induced cardiovascular stress. AM was also shown to have direct cardioprotective effects in reducing ventricular remodelling following MI by acting as an endogenous suppressor of myocyte hypertrophy and fibroblast proliferation (Tsuruda *et al.* 1998, 1999). Nishikimi *et al.* showed AM enhanced fibrosis in AM+/- mice compared with the wild type littermates (Nishikimi *et al.* 2005). Taken together this evidence shows that AM is playing a protective role in the cardiovascular system.

Previously within our group, we have used receptor activity modifying protein 2 (RAMP2) TG mice with vascular smooth muscle targeted RAMP2 over expression (Tam *et al.* 2006) to directly influence functional AM_1 receptor (CL/RAMP2) activity. Our group demonstrated that there was no difference on the basal BP

between wild type (WT) and RAMP2 TG mice. When treating these mice with Ang II (0.9mg/kg/day) for 13 days, a similar hypertensive response occurred in both WT and RAMP2 TG mice. However vascular hypertrophy only developed in the WT mice treated with Ang II (0.9mg/kg/day) for 13 days. The presence of VCAM-1 and MCP-1 on the aortic wall was significantly increased in the Ang II-treated WT mice compared to vehicle-treated mice. However this effect was not seen in RAMP2 TG mice. *In vitro* studies demonstrated that RAMP2 TG VSMCs grew much slower than the WT cells. With the addition of Ang II (10^{-7} M), WT cell number was increased significantly as early as 24 hours but not in RAMP2 TG cells. Furthermore the AM_1 receptor antagonist AM_{22-52} (10^{-7} M) enhanced the proliferation induced by Ang II significantly in the RAMP2 TG cells. This effect of AM_{22-52} was not seen in WT cells. These findings within our group have therefore provided evidence for a protective role for the vascular smooth muscle AM1 receptor, which provide evidence that the protective vascular AM1 receptor may be a novel target for the treatment of vascular hypertrophy. The findings from this study are therefore the basis for the model which is utilised in this thesis.

Intermedin, or also known as adrenomedullin-2 (IMD/AM2) is also a member of the calcitonin family of peptides and shares vasodilatory properties similar to CGRP and adrenomedullin, although at a less potent level (Jolly *et al.* 2009; Bell and McDermott, 2008), although it is less abundantly expressed (Bell and McDermott, 2008). It has been shown to be located in VSMCs of the skin (Kindt *et al.* 2007) and coronary vessels (Morimoto *et al.* 2007), in addition to cardiomyocytes, adipocytes and endothelial cells (Morimoto *et al.* 2007). Due to its vasodilatory action, it is also thought to have cardiovascular protective benefits such as hypotensive actions (Fujisawa *et al.* 2007), thus inhibiting vessel contractility (Dong *et al.* 2005). Similar to the actions of AM and CGRP, intermedin inhibits the progression of vascular hypertrophy and remodelling in terms of fibroblast and VSMC proliferation (Yang *et al.* 2010; Cai *et al.* 2010; Bell and McDermott, 2008). In a study by Zhang *et al.* in 2009 it was shown that intermedin and its receptors were readily upregulated in response to the onset of hypertension in the vascular endothelium in a mouse model of ischaemia and reperfusion (Zhang *et al.* 2009; Bell et al. 2008), and as such, a beneficial and protective role has been suggested for intermedin also in both *in vivo* and *in vitro* endothelial damage (Song *et al.* 2009).

1.14 CGRP receptor antagonists

CGRP antagonists are commonly used when researching the role of CGRP and its receptor. The removal of the ring structure at the CGRP N-terminus results in the generation of the CGRP peptide antagonist. At present, the two most commonly used CGRP antagonists are the peptide fragment $CGRP_{8–37}$, (Chiba *et al.* 1989) and the non-peptide molecule BIBN4096BS (Doods *et al.* 2000). For many years, $CGRP_{8–37}$ was the only available CGRP antagonist. It was identified in rat liver plasma membrane studies, where human CGRP and $CGRP_{8–37}$ were able to bind and displace radiolabelled CGRP binding, but $CGRP_{8–37}$ did not lead to activation of adenyl cyclase (Chiba *et al.* 1989). The non-peptide antagonist BIBN4096BS was generated by Boehringer and was found to behave as a competitive antagonist. It showed ability to shift concentration response curves of CGRP induced cAMP formation to the right, and had no agonist properties in concentrations up to 100μM (Doods *et al.* 2000). This antagonist is more potent than $CGRP_{8–37}$, but is considered to be relatively human selective, as it is much less potent in the rat (Doods *et al.* 2000; Wu *et al.* 2000). However, this antagonist was shown to be more effective than $CGRP_{8–37\ in}$ both the mouse mesentery and aorta *in vitro* (Tam and Brain, 2004). It is well known that CGRP plays a pivotal role in migraine (Goadsby *et al.* 1990). High serum levels of CGRP correlate with an increased incidence of migraine attacks (Juhasz *et al.* 2003). Subcutaneous injection of BIBN4096BS in migraine patients caused reduction of symptoms (Olesen *et al.* 2004 and Russo, 2007). These findings propose this class of antagonist as a potential novel therapeutic compound for migraine and associated diseases such as cluster headache (Goadsby and Edvinsson, 1994).

The most recently identified antagonist to date is named Telcagepant (formerly MK-0974, Merck). Telcagepant has been involved in clinical trials as an anti-migraine drug and reached phase III of clinical trials before being aborted due to side effects of minor liver abnormalities (Edvinsson and Linde, 2010). Pain associated with migraine was reduced in patients using these CGRP blockers due to the compounds ability to block CGRP receptors on the trigeminal and central nervous systems, and also possibly the vasculature. However whilst serotonin (5-HT)1B/1D receptor agonists (triptans) are considered the best treatment for migraine at present, the CGRP antagonists such as Telcagepant do not cause vasoconstriction of the coronary (Chan *et al.* 2010), cerebral and middle

meningeal arteries and therefore may be notably safer for use in patients with cardiovascular problems (Edvinsson *et al.* 2005). An important aspect to consider about this compound is that it was available orally unlike BIBN4096BS. Other CGRP antagonists are thought to be in development for use as anti-migraine agents. It is anticipated that these compounds, if they can overcome developmental hurdles, will provide an improved treatment for migraine.

1.15 The CGRP receptors

Two distinct forms of CGRP receptors were originally identified in the late 1980s through pharmacological studies, these being $CGRP_1$ and $CGRP_2$. $CGRP_1$ was considered to be the predominant mediator of cardiovascular effects (Dennis *et al*. 1990). This receptor classification was distinguished by their sensitivity to $CGRP_{8-37}$ in a range of tissue preparations as reviewed by Hay (2007) and Brain and Grant (2004). This antagonist showed preference for the blockade of the $CGRP_1$ receptor in the guinea pig atria and in vascular tissue, whereas $CGRP_{8–37}$ was less effective at antagonising the effects of CGRP in the rat vas deferens, a predominant site for $CGRP_2$ receptors (Dennis *et al.* 1990; Quirion *et al.* 1992; Chiba *et al*. 1989). Linearized CGRP analogues such as diacetoamidomethyl cysteine CGRP {[Cys(ACM)2,7]hCGRP} which is formed by reduction of the disulfide bond of CGRP, show preferential agonist potency for the $CGRP_2$ receptor (Doods *et al.* 2000). It was documented that receptors antagonized by $CGRP_{8-37}$ with an approximate p*K*b value of 7.0 were classed as $CGRP_1$ receptors, whereas those with values less than 6 were classified as $CGRP_2$ (Poyner, 1995; Quirion *et al.* 1992). However these classifications were then debated by Poyner and Marshall who suggested that mean pA2 values ranging between 5-8.1 in 11 different tissues from the rat do not allow these receptors to be accurately divided into two groups (Poyner and Marshall, 2001). The $CGRP_2$ receptor phenotype is now believed to be due to receptors for other peptides which show significant affinity for CGRP such as AM_2 and $AMY_{1(a)}$, and therefore the $CGRP_2$ receptor term is of historical significance (Hay *et al.* 2008).

The calcitonin receptor-like receptor (CLR) belongs to the family of GPCRs, and was first cloned in 1993 (Chang *et al.* 1993; Nujki *et al.* 1993). Evidence for its role in CGRP activity was shown by Aiyar *et al.* (1996), in which CGRP binding and

cAMP accumulation was observed in cells. A major breakthrough in $CGRP_1$ receptor function was shown by McLatchie *et al.* (1998), where they demonstrated that the CLR required co-binding of receptor activity modifying proteins (RAMP) in order to form a functional $CGRP_1$ receptor on the cell surface. This CLR/RAMP1 complex is highly sensitive to $CGRP_{8-37}$. RAMPs are single transmembrane proteins, forming three distinct types, RAMP1, RAMP2 and RAMP3. CLR linked with RAMP1 leads to a high affinity CGRP receptor and in some cases an intermedin (IMD/AM2) receptor. CLR linked with RAMP2 forms a functional adrenomedullin/intermedin receptor, whilst CLR linked with RAMP3 forms an IMD/AM2 receptor, and a second receptor for AM. This receptor can be activated by IMD/AM2 with high potency, in addition to also responding well to CGRP (Hong *et al.* 2012; Brain and Grant, 2004, Figure 1.7). In addition to these components, immunoprecipitation studies have identified the requirement of an intracellular peripheral membrane protein, named receptor component protein (RCP) for the amplification of signal generated by CGRP receptor activation and signal transduction at the GPCR (Prado *et al.* 2001). Molecular knockdown techniques have shown that blockade of RCP results in decreased cAMP production, thus providing evidence to suggest that this protein is of key importance for a fully functional receptor (Evans *et al.* 2000). There is a range of evidence to show that CLR and the RAMPs are localised to the vasculature, in particular the smooth muscle cells, as shown by immunohistochemistry (Oliver *et al.* 2002; Edvinsson *et al.* 2010; Chan *et al.* 2010; Albertin *et al.* 2010). CLR mRNA in human microvascular endothelial cells has been shown to be upregulated in hypoxia (Nikitenko *et al.* 2006). Other sources of these receptors include coronary arteries (Hasbak *et al.* 2003), cardiomyocytes and fibroblasts (Nishikimi and Matsuoka, 2005) in addition to both macro and microvascular endothelial cells (Hagner *et al.* 2003). It has been noted recently that RAMPS are able to function independently of CLR with other receptors which create amylin (Udawela *et al.* 2004), secretin (Harikumar *et al.* 2009) and the vasoactive intestinal peptide (VIP), pituitary adenylate cyclase-activating polypeptide (PACAP), VIP/PACAP receptors (Sexton *et al.* 2006).

The different components of the CGRP receptor are now each discussed in turn.

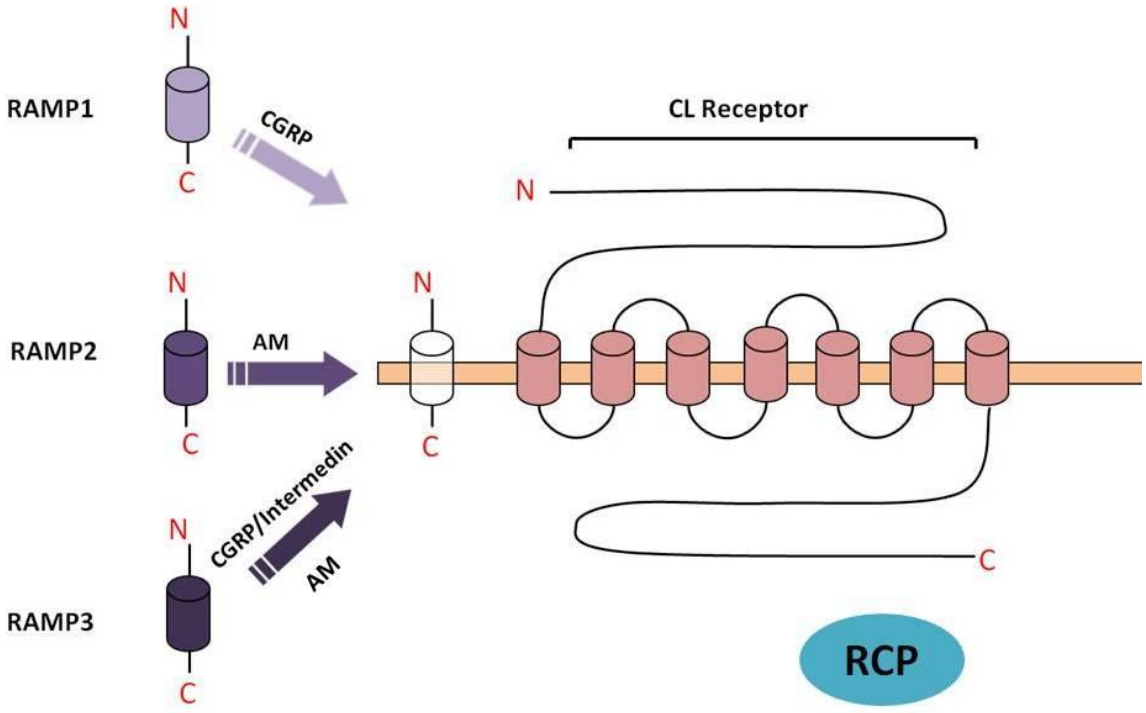


Figure 1.7. Structure of the CGRP receptor. Heterodimerization of CL with RAMP1 leads to the formation of a functional CGRP receptor (and in some cases Intermedin) on the cell surface, whilst association with RAMP 2 forms an AM1/2 receptor and with RAMP 3 an Intermedin/CGRP/AM receptor is formed. The addition of the intracellular peripheral membrane protein, named receptor component protein (RCP) is required for signal transduction at the GPCR.

1.16 Calcitonin receptor-like receptor (CLR)

CLR belongs to the family of type II GPCRs (Sexton *et al.* 2009). Its structure consists of a seven transmembrane domain protein coupled intracellularly to a G-protein. It shares 55% homology with the calcitonin receptor (Fluhmann *et al.* 1995). There is only a two amino acid difference in length between the human and mouse CLR (mouse 463 amino acids, human 461 amino acids), sharing about 89% homology.

Kuwasako *et al.* (2003) identified the importance of cysteine residues in the extracellular loops of human CLR. Substitution of these residues with alanine in the first two loops caused a decrease in cAMP production. They show that these cysteines form a disulphide bond which is important for ligand binding and activation (Kuwasako *et al.* 2003). In addition, a more recent study by Banerjee *et al.* also characterised specific leucine residues on the CLR N-terminus which participate in the high affinity CGRP binding, but not activation of the mature CGRP receptor, named CGRP-F37 (Banerjee *et al.* 2006). Together, these studies prove that the CLR has specific areas which are important and required for the migration of the CLR to the cell surface in order to form a functional receptor. Studies have also shown that there are regions extending from the transmembrane domains 1 and 5 are necessary for the transport of RAMP to the plasma membrane. In addition to this, the third intracellular loop on the N-terminus is specific for cell surface relocation of RAMP2, but not RAMP1 or RAMP3 (Kuwasako *et al.* 2009).

1.17 Receptor activity modifying proteins (RAMP)

The RAMP family consists of three single transmembrane proteins (between 147 and 189 amino acids). They have a short intracellular C-terminal of $\sim$10 residues and long extracellular N-terminus of $\sim$100 amino acids, which may be involved in determining the eventual phenotype of the receptor and ligand binding, (McLatchie *et al.* 1998; Zumpe *et al.* 2000; Qi and Hay, 2010). The C-terminus is suggested to be important in the RAMP-receptor interactions (Udawela *et al.* 2006; Qi and Hay, 2010). Discovered in 1998 by McLatchie *et al.* it was shown that the CLR protein must be expressed as a dimer with a RAMP in order to form a functional receptor at the cell surface as discussed previously. However it is

now known that both the CLR and RAMPs can move to the cell surface independently, although they do not function alone as a CGRP receptor (Flahaut *et al.* 2003; Qi and Hay, 2010). The RAMP family regulate the transport of the CLR, however as they do not possess any functional activity, they are therefore not CGRP receptors by themselves (McLatchie *et al.* 1998).

1.18 Vasodilatation by CGRP

CGRP is the most potent microvascular vasodilator identified to date (Brain *et al.* 1985). It has potency ~10 fold greater than the most potent prostaglandins, and is 10–100 times more potent than vasodilators such as acetylcholine and substance P. In skin, picomole amounts of injected CGRP were able to induce reddening caused by resistance vessel vasodilation at the microvascular level (Brain *et al.* 1985). CGRP also has a particularly long duration of action in comparison to other vasodilators. Studies have shown that a picomole (pmol) dose injected into the human skin produced an erythema which lasted between 5 and 6 hours (Brain *et al.* 1986). The 37 amino acid neuropeptide has been shown to selectively cause potent dilation in multiple vascular beds, including the cerebral, coronary and kidney vascular beds, as mentioned in the introduction (Brain and Grant, 2004).

The vasodilator effect of CGRP has been studied extensively *in vivo*. In humans CGRP is a vasodilator when given systemically (Gennari and Fischer, 1985). Intravenous administration (IV) of CGRP into rodents causes hypotension, and positive inotropic and chronotropic responses in the heart (Ando *et al.* 1990 and Gardiner *et al.* 1991). It is thought that these cardiac effects are due to reflex sympathetic nervous activity in an attempt to compensate the hypotension, and also a direct action on the cardiac muscle (Ando *et al.* 1990). In contrast, intracerebroventricular injection of CGRP causes a hypertensive response, by activating the sympathetic nerves and releasing noradrenaline (Fisher *et al.* 1983). Despite these actions, the majority of studies using antagonists and knockout models suggest that CGRP does not have a pivotal role in physiological control of systemic BP in normal individuals, as the deletion or blockade of CGRP generally fails to affect it. Injection of the selective CGRP receptor antagonists BIBN4096BS/$CGRP_{8-37}$ does not affect resting heart rate or BP in a range of species, including rats (Arulmani *et al.* 2004; Zeller *et al.* 2008) and humans (Olesen *et al.* 2004). BIBN4096BS has been tested in phase II clinical trials as a potential novel treatment (Doods, 2001) and found to be effective against the symptoms of migraine, without significant acute side effects (Edvinsson, 2003).

The potency of CGRP as a microvascular vasodilator and its distribution throughout the peripheral nervous system suggests that it may normally have a greater impact at a more local level, regulating vascular responsiveness and protecting organs from injury. The potential importance of CGRP in this respect is demonstrated by conditions in which CGRP signalling is absent, or inappropriately enhanced. Destruction of rat sensory nerve fibres by neonatal treatment with capsaicin leads to a loss of cutaneous neuropeptides, such as CGRP. These animals commonly suffer from skin lesions as a result (Thomas *et al.* 1994). In comparison to this, increased or inappropriate CGRP release has been linked to facial reddening in blushing syndromes (Wyon *et al.* 1998) and as previously discussed the onset of migraine (Goadsby *et al.* 1988).

In terms of beneficial effects, these are linked to its ability to dilate blood vessels, increasing blood flow and tissue perfusion. This effect is therefore believed to enhance and promote wound healing, as it has previously been shown that depletion of the sensory nerves decreased the survival of skin flaps in the rat, whereas treatment with CGRP enhanced flap survival and improved blood flow (Kjartansson and Dalsgaard, 1987). There is also good experimental evidence that CGRP can protect against the damage caused in tissue ischemia (Kallner, 1998; Uren *et al.* 1993; Chai *et al.* 2006; Li *et al.* 2008), although mechanisms involved in its release are unclear.

CGRP exhibits its most potent activities in the microvasculature, such as facial flushing (Gennari and Fischer, 1985). Facial flushing is also seen following IV administration in humans when CGRP is administered at lower doses than those which affect systemic BP. This suggests that the microvasculature is more sensitive to CGRP than the intact vascular system and that the local, rather than systemic effects of CGRP may be important in BP regulation.

Vascular relaxation and vasodilatation is mediated via activation of the CGRP receptor (CLR/RAMP1) which can be blocked by the antagonist $CGRP_{8–37}$. Several mechanisms involved in CGRP mediated relaxation have been identified. These mechanisms include either NO-dependent endothelium-dependent mechanisms or cAMP-mediated endothelium-independent pathways (Brain and Grant, 2004). The most common pathway is NO- and endothelium-independent. CGRP administration triggers a rise in cAMP. In the absence of endothelium,

CGRP is able to cause relaxation, suggesting it must directly act on the smooth muscle cells to stimulate adenylate cyclase (AC). This has been shown in cultured smooth muscle cells, although high doses were required (Crossman *et al.* 1990), endothelium denuded arteries in cat brain vessels (Edvinsson *et al*. 1985) and in human intracranial arteries (Edvinsson *et al.* 1998). This resulting rise in cAMP then activates protein kinase A (PKA), which phosphorylates and opens up potassium (K+) channels, thus leading to relaxation. These potassium channels are adenosine triphosphate (ATP)-sensitive. In fact, the use of the ATP sensitive K + channel blocker, glibenclamide blocked the CGRP induced response, and hyperpolarizes arterial smooth muscle (Nelson *et al.* 1990). Endothelium-dependent vasodilatation is mediated through an increase in both cAMP and cyclic guanosine monophosphate (cGMP). The relaxation is dependent on the presence of endothelium. This causes the release of NO, which relaxes the smooth muscle cells by activation of guanylate cyclase and the accumulation of cGMP (Gray and Marshall, 1992).

Recently a direct interaction between ET-1 and CGRP has become apparent. ET_A receptors are expressed on sensory motor nerves alongside CGRP (Wang *et al. 2006*), which in turn activates AC (Brain and Grant, 2004). In a study by Meens *et al.* it was shown that ET-1 induced arterial contractions were reversed and therefore sensitive to relaxation by the administration of CGRP in a specific manner (Meens *et al.* 2009).

1.19 CGRP and its vascular protective mechanisms

As previously discussed, CGRP is the most potent vasodilator to date (Smillie and Brain, 2011), with its vasodilator activity predominantly mediated by CGRP binding to its receptor on the membrane of both VSMCs and the endothelium of blood vessels. This then stimulates either endothelium dependent or independent mechanisms of vasodilatation (Brain and Grant, 2004). CGRP-immunoreactive fibres have been reported to innervate the vasculature, particularly in the myocardium and coronary vessels (Ieda *et al.* 2006), thus a cardioprotective role for CGRP has been suggested. αCGRP/calcitonin knockout (KO) mice have been reported to have increased susceptibility to ischaemia/reperfusion injury. This coincides with increased vascular cell damage and an increase in ROS

generation (Huang *et al.* 2008). Recovery following this ischaemia/reperfusion injury is also blunted in mice lacking the αCGRP gene due to a loss of the vasodilator mechanism (Huang *et al.* 2008). In cultured rodent cardiomyocytes, CGRP has been reported to inhibit apoptosis of the cells after induction of the catecholamine neurotransmitter; norepinephrine (Zhao *et al.* 2010), suggesting a protective role for CGRP. Also, diabetic mice are less able to generate cardioprotection via ischaemic preconditioning, with this thought to be due to a loss of CGRP activity. Administration of CGRP into these rodents was shown to improve the cardioprotective ability of the subjects (Zheng *et al.* 2011).

Hypertension and cardiovascular disease display both increased BP and the progression of vascular remodelling and inflammation. During this remodelling, there is an increase in the rate of hypertrophy and proliferation of the VSMC within the vessel wall. Endothelium dysfunction also occurs, and subsequently, NO production is impaired. CGRP has been shown previously to be protective in the onset of cardiovascular disease in a variety of studies. Overexpression of RAMP1 in RAMP1 transgenic mice protects against Ang II induced endothelial dysfunction through the increase of CGRP-induced vasodilatation, shown previously by Chrissobolis and co workers when studying vascular responses to CGRP in carotid and basilar arteries *in vitro* (Chrissobolis *et al.* 2010). CGRP stimulation has also been shown to reduce the numbers of migrating neutrophils and monocytes through the endothelium. There was also a decrease in chemokine expression through the inhibition of the NFκB (contributes to atherosclerotic lesion formation) pathway by this CGRP stimulation in cultured endothelial cells (ECs, Huang *et al.* 2011). Endothelial progenitor cells (EPCs) play a critical role in vascular endothelial repair, and a decline in EPC content is thought to predispose subjects to future cardiovascular complications. CGRP synthesis has recently been discovered in EPCs and this EPC derived CGRP has been shown to be protective against EPC senescence induced by Ang II (Zhou *et al.* 2010). To further emphasise the protective importance of EPC derived CGRP, VSMC hypertrophy has also been shown to be attenuated in Ang II induced hypertension (Fang *et al.* 2011). This inhibitory effect of CGRP in VSMC proliferation has also been shown in *in vitro* studies using the Ang II stimulus (Qin *et al.* 2004). Furthermore, when administering CGRP derived EPCs to subjects, lowered vascular resistance results alongside inhibited VSMC proliferation and thickening

of the vessel wall (Zhao *et al.* 2007). In addition to this, CGRP activated endothelial cells have been shown to significantly reduce the number of inflammatory mediators such as monocytes and neutrophils which were recruited to the vessel wall in cultured human dermal microvascular endothelial cells (Huang *et al.* 2011). This therefore provides evidence for a protective role of the CGRP signalling pathway in the onset of vascular inflammation. Manipulation of this specific signalling pathway could prove to be a therapeutic target in the prevention of hypertension induced vascular inflammation; however the mechanisms in which it plays this role are less well understood.

1.20 CGRP and its role in hypertension evidence for a role of CGRP in the vascular component in hypertension: Human and rodent studies

The role of CGRP in human studies and experimental models of hypertension in the rodents provide key evidence that CGRP is protective in vascular inflammation and in cardiovascular diseases. The development of the CGRP deficient mouse has also deepened our knowledge of the role of CGRP in models of hypertension and the onset of inflammation and oxidative stress. These models will also be discussed in detail.

1.20.1 Human studies

The perivascular location of neuronal CGRP makes it ideally situated to be released to exert local effects, with little reaching plasma. Circulating CGRP levels are elevated in postmenopausal women with facial flushing in comparison to premenopausal subjects (Gupta *et al.* 2008). In addition, in an experimental situation, infusion of Ang II to normotensive humans causes dose-dependent increases in plasma CGRP levels, in parallel with an increase in BP induced by Ang II (Portaluppi *et al.* 1993). Furthermore, plasma CGRP concentrations are also increased in response to the elevated arterial BP in patients with secondary hypertension (Masuda *et al.* 1992). The increase in CGRP levels have also been shown in experimental models comparing CGRP levels in normotensive subjects to patients and models with essential and secondary hypertension (Li *et al.* 2009). During exercise CGRP levels progressively increase in both normotensive and hypertensive patients however there are no significant differences in the circulating CGRP levels between these groups of patients (Lind *et al.* 1996). Low to normal levels of circulating CGRP has been observed in acute hypertension (Edvinsson *et al.* 1989; Schifter *et al.* 1991; Portaluppi *et al.* 1992, see Table 1.1), and in severe hypertension it has been shown that there is a significant correlation with systolic and diastolic BP and subsequent circulating CGRP levels (Edvinsson *et al.* 1992).

These findings suggest that modifications of plasma CGRP concentrations are likely to be a normal response to vasomotor changes, in which CGRP may play a potential role in the regulation of peripheral vascular tone and BP in humans. It is therefore perhaps surprising that the majority of studies from patients with essential hypertension show that plasma CGRP levels are reduced compared to normal volunteers (Portaluppi *et al.* 1992; Tang *et al.* 1989; *Wang et al.* 2007, see Table 1.1). This has been recently suggested to be directly related to the role of CGRP in the pathophysiology of essential and/or spontaneous hypertension, possibly because CGRP release is enhanced early in pathogenesis to try and counteract the onset of hypertension; it then becomes downregulated as the hypertension progresses. Interestingly, there is evidence that hypertensive patients have a selectively inhibited response to exogenous CGRP in the cutaneous microvasculature *in vivo* (Lindstedt *et al.* 2006). This suggests a defect in the CGRP pathway may occur. On the other hand, an enhanced vasodilator response to CGRP was observed in the subcutaneous arteries of hypertensive patients (Lind and Edvinsson, 2002). Moreover, there is also evidence to state that plasma CGRP levels are unchanged in hypertensive patients (Schifter *et al.* 1991). These different findings may be due to variations in choices of treatment and sampling methods or in the specific nature of the hypertension in the patients studied. At present the effect of CGRP antagonists in patients with hypertension is unknown.

CGRP antagonists, as discussed earlier, are in development for the treatment of migraine. If CGRP plays a role in protecting against the onset of hypertension, it is important to assess the cardiovascular risk associated with administration of CGRP antagonists. It may be assumed that if migraine treatments are given on an occasional basis, when the onset of migraine is detected in a normotensive healthy patient with a relatively low incidence of attacks, then the vascular risk may be small. However, if the patient suffers more frequent attacks and wants to take the medication more frequently, the risk in terms of adverse cardiovascular events may be more difficult to assess due to the lack of understanding of the total role of CGRP in both migraine and BP control. This problem has been questioned by a number of groups, looking first for effects of $CGRP_{8–37}$ (Chiba *et al.* 1989) and more recently using non-peptide antagonists in animal models (Doods *et al.* 2000). Most recently it was shown that the Merck antagonists

Telcagepant did not induce contraction or relaxation of human coronary vessels, i.e. a neutral effect, under conditions where a (5-HT)1B/1D agonist triptan, used in migraine treatment and known to possess an adverse effect of coronary vasospasm, did (Chan *et al.* 2010; Regan *et al.* 2009). These results build upon a body of evidence, suggesting that the CGRP antagonists should be safer than the triptans in mediating adverse side effects in the heart.

Plasma CGRP levels					
Source	**Normotensive**	**Hypertensive**	**Comments**	**Study Design**	**Sample Preparation**
Tang et al., 1989	33pg/ml	2.3pg/ml	Plasma CGRP levels decreased in hypertensive patients.	Essential hypertensive patients	
Edvinsson et al., 1989	132.5pg/ml	94pg/ml	Plasma CGRP levels decreased in hypertensive patients.	Severe hypertensive patients with constant high BP. 17 patients with BP>204/127 mmHg matched with normotensive controls	
Schifter et al., 1991	36±9pmol/l (133.2±12pg/ml)	37±7pmol/l (137±25pg/ml)	No sig. change in plasma CGRP levels	Patients with moderate-sever hypertension, based on BP.	Un-extracted plasma
Portaluppi et al., 1992	12.1-17.7pmol/l (44.7-65.49pg/ml)	5.6-12.8pmol/l (20.7-47.3pg/ml)	Plasma CGRP levels decreased in hypertensive patients.	Essential hypertensive patients, being treated with calcium channel blockers, however treatment was discontinued 2 weeks prior to study. 10 patients, 4 male and 6 female, 26-42 years.	RIA kit from Peninsula Lab Inc. (Belmont, USA) after extraction of the peptide from plasma using Sep-Pack column method
Masuda et al., 1992	18.2±1.8pg/ml	Essential: 31.2±3.9pg/ml Secondary: 45.8±7pg/ml	Plasma CGRP levels increased in hypertensive patients.	*Normotensive:* 14M, 4F mean±sem age= 40.4±3.4yrs. *Essential:* 9M, 7F, 50.6±2.7yrs. *Secondary:* primary aldosteronism- 6M, 4F, 47.3±5.8yrs. Patients were taking no medication and restricted to a controlled diet for the duration of the study (120nmol/day sodium).	RIA system established in-house. Plasma samples extracted using column method
Lemne et al., 1994	21.7±8.7pmol/l (80.2±32pg/ml)	24.2±8.6pmol/l (89.5±31.8pg/ml)	No sig. change in plasma CGRP levels	Borderline essential hypertension (constant diastolic BP on the borderline hypertensive range over a 3 year period). 81 males with borderline hypertension matched with 80 normotensive controls.	Reverse-phase HPLC performed for plasma purification and the RIA performed using ant-serum CGRP Ab raised in conjugated rat CGRP
Dong et al., 2006	3.8±0.4pmol/l (14.0±1.4pg/ml)	. 3±0.34pmol/l (8.5±1.2pg/ml)	Plasma CGRP levels decreased in hypertensive patients.	Pre-eclamptic hypertensive patients. Normotensive: N=28 13-16wk, 18-22wk gestation N=6 23-32wk, 36-40wk gestation N=8 *Pre-eclampsia:* N=8, diagnosed on basis of development of hypertension with BP>140/90mmHg on 2 separate occasions and no prior history of hypertension.	Plasma extraction using column method (Sep-Pack C18 cartridges). RIA- using CGRP RIA kit (Belmont, CA)
Li et al., 2009	45pg/ml	28pg/ml	Plasma CGRP levels decreased in hypertensive patients.	20 male patients newly diagnosed with essential hypertension matched with 22 healthy controls, 48-65yrs. Hypertension diagnosed on a basis of BP of 140-170 systolic and 90-99 diastolic.	

Table 1.1: Comparison of plasma CGRP levels in patients with essential and secondary hypertension. (Please note that it is important to be aware that differing results between studies may be due to different sample preparation)

1.20.2 The development of the genetically modified αCGRP KO mouse and the role of CGRP in BP maintenance

The development of genetically modified mice has led to the study of αCGRP deletion in a range of techniques, depending on the mouse and laboratory studied as illustrated in Table 1.2. There are conflicting views in the literature depending on the type of KO mouse used. Lu *et al.* originally showed in 1999 that genetic deletion of αCGRP does not influence BP, either basally after exercise, or after phenylephrine infusion. On the other hand Gangula *et al.* (2000) and Li *et al.* (2004) demonstrated an increase in basal BP and renin-angiotensin activity in combined calcitonin/αCGRP KO hypertensive mice. However the additional deletion of calcitonin (CT) could be responsible for this. Oh-Hashi *et al.* (2001) and Kurihara *et al.* (2003) then studied a male 129/Sv x C57BL/6 hybrid αCGRP knockout mice that had increased MAP and heart rate. This response was suggested to be linked to raised sympathetic activity as it was blocked by the alpha-adrenergic antagonist prazocin and the cholinergic antagonist atropine. The range of different findings with the different αCGRP KO mouse strains are surprising. For ongoing research into CGRP, it would seem important to choose a model similar to that of Lu *et al.* (1999) which shows no baseline MAP difference, holding only deletion of αCGRP, and not the calcitonin gene. Based on our understanding of the role of CGRP in humans this would appear important for the modelling of the role of CGRP in human hypertension.

Bolus administration of α-CGRP results in a rapid decrease MAP in both WT and αCGRP KO mice, with an increased response in the αCGRP KO mice, resulting in a further decrease in MAP. Results like these suggest that the lack of αCGRP increases the responsiveness to CGRP in the vasculature possible through a receptor–mediated mechanism (Gangula *et al.* 2000). This increase in vascular sensitivity to CGRP has also been noted in SHR (Wimalawansa, 1996).

RAMP1 deficient mice exhibit increased BP in comparison to WT mice. Administration of αCGRP causes potent relaxation of the arteries in the WT group and suppresses the production of pro-inflammatory cytokines in Lipopolysaccharide (LPS)-induced inflammatory responses. These suppressor mechanisms however are not observed in RAMP1 deficient mice, indicating that

CGRP signalling through the CLR/RAMP1 receptor plays a crucial role in BP regulation and suppression of inflammatory mediator generation (Tsujikawa *et al.* 2007). In a more recent study, upregulation of RAMP1 has also been shown to enhance the antihypertensive actions of endogenous CGRP in RAMP1 transgenic mice infused in a chronic manner with Ang II (Sabharwal *et al.* 2010). Although it is widely supported that the antihypertensive actions of CGRP are due to its role as a vasodilator, other mechanisms may also be involved. This particular study showed that CGRP can be implicated in the modulation of baroreflex sensitivity, in which the baroreceptor reflex is a major regulator of arterial BP as previously discussed, (Sabharwal *et al.* 2010). The role of the baroreflex is to buffer fluctuations in BP by responding to reflex changes in heart rate and vascular resistance e.g. High baroreflex sensitivity results in lowered BP in comparison to low baroreflex sensitivity which results in an increase in mean arterial pressure and subsequently an increase in cardiovascular risk (Tatasciore *et al.* 2007). Thus CGRP may provide additional protection in the onset of hypertension.

A study by Supowit *et al.* attempted to identify the role of αCGRP in hypertension by uninephrectomy, DOCA-salt administration, and 0.9% saline drinking water in WT and calcitonin/αCGRP KO mice. This treatment produced a significant 35% MAP increase in both WT and KO mice. Heart and kidney analysis from the hypertensive WT mice showed no pathological changes compared with their normotensive controls. However vasculitis was observed in hearts of the hypertensive KOs, characterised by thickening and inflammation of the vessel walls. In addition to this, the kidneys of these mice exhibited prominent glomerular changes such as mesangial and crescent proliferation. The data from this study suggests that deletion of αCGRP makes the heart and kidneys more vulnerable to hypertension-induced end organ damage, and thus indicating a protective role for this peptide (Supowit *et al.* 2005).

In a later study by the same group, Bowers and co workers again attempted to investigate the role of αCGRP in DOCA-salt hypertension-induced renal damage with the use of the calcitonin/αCGRP KO mouse. Although these mice had an increased BP at basal levels, there was no morphological or functional changes in the kidney under normal conditions. However these mice showed exacerbated hypertension and renal damage in response to DOCA-salt treatment when compared to WT mice at both 14 and 21 days, although they were unable to

identify key mechanisms by which this occurred. They concluded that it was not possible based on these findings to elucidate whether the changes observed were dependent on an increase in BP, or whether it was αCGRP dependent (Bowers *et al.* 2005).

Source and Origin	Genotype/strain/age/gender (if stated)	Purpose of the experiment	Effect on BP	Comments
Lu *et al.,* 1999	C57BL/6	Examine basal BP, after exercise or phenylephrine infusion	No effect	KO mice generated in which αCGRP but not calcitonin was deleted.
Li *et al.,* 2004	3-6month male WT and αCGRP/CT KO C57BL/6	Test effect of αCGRP KO on BP and RAS activity	Basal MAP sig. greater in the αCGRP/CT KO	Increase in circulating RAS activity in the αCGRP/CT KOs. Combined calcitonin and CGRP KO.
Gangula *et al.,* 2000	129/C57 genetic	Role of CGRP in the long term regulation of CV function, including hypertension. BP recorded under terminal anaesthesia	Increased baseline MAP in αCGRP KO	Also reported increase in renin-angiotensin activity. Combined calcitonin and αCGRP KO could be the explanation for this. Combined calcitonin and αCGRP KO.
Supowit *et al.,* 1997	C57BL/6	Role of CGRP in DOCA salt-induced hypertension	Increased baseline MAP in αCGRP KO	Combined calcitonin and αCGRP KO.
Oh-Hashi *et al.,* 2001	WT and αCGRP KO 129/sv_C57BL/6 Males	Role of CGRP in sympathetic nervous system. BP recorded on conscious and anaesthetised mice.	Increased baseline MAP and HR in αCGRP KO	Combined calcitonin and αCGRP KO. Due to increased sympathetic activity.
Kurihara *et al.,* 2003	WT and αCGRP KO mice	Physiological role of αCGRP. BP recorded on conscious and anaesthetised mice.	Increased baseline MAP and HR in αCGRP KO	Targeting of αCGRP gene was designed to reserve the exons encoding the precursor for calcitonin. KO mice generated in which αCGRP but not calcitonin was deleted.

Table 1.2: Comparison of the different types of generated CGRP KOs and subsequent effects on blood pressure.

1.20.3 Rodent studies

The role of CGRP in experimental animal models of hypertension is under study, with developing evidence suggesting that CGRP activity is important in resisting the onset of hypertension and vascular dysfunction (Smillie and Brain, 2011). It is known that CGRP and its receptor are expressed at elevated levels in experimental models of hypertension; however it has been suggested that the role of CGRP depends on the experimental model chosen (Deng and Li, 2005).

As discussed previously, studies using the KO mouse have shown that sole deletion of αCGRP does not influence normal baseline BP; therefore it can be deemed reasonable to say that αCGRP plays a minor role in the regulation of baseline BP. This has also been confirmed in human studies where injections of both receptor antagonists BIBN4096BS and $CGRP_{8–37}$ had no effect on both heart rate and systemic BP at basal levels (Arulmani *et al.* 2004 and Olesen *et al.* 2004). It therefore may be possible to believe that CGRPs functionality as a hypotensive and vasodilatory agent is more pronounced in the microcirculation of specific tissues, and only plays a role in the onset of cardiovascular disease due to a specific stimulus such as RAS over activation and the inflammatory response in different models of hypertension.

However, whilst circulating plasma CGRP levels are considered to be low in humans under normal conditions, it has been shown that these levels are lower in essential hypertensive patients as previously mentioned. The role of the microvasculature, especially resistance arteries, is important for the regulation of BP and tissue blood flow. (Mulvany and Aalkjaer, 1990) An increased total vascular resistance contributes to hypertension (Zimmerman, 1983). Early studies with CGRP in the rat suggested that CGRP, derived from sensory nerves, plays an important role in the mesentery via an interaction with sympathetic nerves (Kawasaki *et al.* 1988). CGRP release and activity has also been shown to be reduced when angiotensin levels are raised (Kawasaki *et al.* 1998). The function of CGRP nerves additionally is suggested to decrease with age, as observed from studies in SHRs (Kawasaki, 2002). However, the mechanisms underlying these pro-hypertensive effects are unclear. A study which aimed to discover the role of endogenous CGRP and the receptor components in the development of Ang II

induced hypertension showed that Ang II infusion over 10 days in seven week old male Wistar rats caused increased mRNA CLR and RAMP1 expression in the mesenteric vessels, which was pressure dependent (Li and Wang, 2005).

Systemic administration of CGRP decreases BP in normotensive and hypertensive rats, (Gangula *et al.* 1999; Itabashi *et al.* 1988; Wimalawansa, 1996 and Preibisz, 1993). This is thought to be mediated through peripheral arterial NO-dependent and independent dilation mechanisms (Itabashi *et al.* 1988).

Gangula *et al.* reported previously that CGRP reversed hypertension induced by NO inhibition in pregnant rats through a possible progesterone dependent mechanism. They then progressed further to identify whether this was indeed hormone mediated or not. In this study they showed that using doses of CGRP (9-360 pmol/kg body weight) resulted in a dose dependent decrease in MAP. This response was significantly greater in pregnant rats compared to non pregnant animals, thus showing that the decrease in vascular tone observed during pregnancy may be mediated in part by a sex steroid hormone-induced increase in the vascular sensitivity to CGRPs vasodilatory action (Gangula *et al.* 1999). In Wistar rats, CGRP (CGRP, 0.1 and 1.0 nmol/kg per min) dose-dependently lowered mean arterial BP and increased the heart rate throughout a 30 minute infusion period. In addition to this, CGRP significantly attenuated the pressor responses to Ang II (100ng/kg per min). In the same study, this group also reported that a higher dose of CGRP (1.0 nmol/kg per min) almost abolished the pressor action of Ang II, and a much higher dose of Ang II (1000 ng/kg per min) was needed to restore the pre-infusion pressure. Plasma renin activity was also dose-dependently increased by CGRP, but this activity was attenuated by simultaneous Ang II infusion (Itabashi *et al.* 1988).

Global overexpression of RAMP1 in mice has been shown to significantly attenuate Ang II induced hypertension and improve baroreceptor reflex activity (Sabharwal *et al.* 2010). The TRPV1 agonist Rutaecarpine has also been shown to possess an anti-hypertensive effect due to the increased CGRP release into the circulation (Deng *et al.* 2004) in SHRs, 2 kidney, 1-clip (2K1C) and renal phenol injury models of hypertension (Deng *et al.* 2004; Qin *et al.* 2007; Li *et al.* 2008). CGRP released by Rutaecarpine treatment has also been shown to reduce platelet aggregation through inhibition of platelet-derived tissue factor (Li *et al.*

2008). The consumption of dietary capsaicin has been suggested to be beneficial in lowering BP and improving endothelial function due to increased vasodilatation by CGRP. Therefore TRPV1 agonists including capsaicin and Rutaecarpine may prove to be novel targets in the treatment of hypertension, thus posing a protective role for CGRP (Yang *et al.* 2010; Hao *et al.* 2011). Recently an electrophysiological and immunohistochemical study provided evidence that a distinct population of sensory peptidergic nerves may play a role in renal pathophysiology, as they are more sensitive to TRPV1-mediated acid stimulation (Ditting *et al.* 2009). In addition, there is some new evidence that the cannabinnoid TRPV1 agonist anandamide regulates CGRP generation and activity in a rodent model of hypertension, with the anandamide transporter acting as a limiting factor (Li *et al.* 2009).

In a 2K1C renovascular hypertension model in the rat, BP was significantly elevated 10 days post operation, however, further treatment with capsaicin to deplete the neurotransmitters within the sensory nerves, causing the BP to elevate further. Mesenteric mRNA CGRP expression was significantly increased, thus this increased production of CGRP is acting as a compensatory dilatory mechanism to counteract the increase in BP in this model. The mechanisms involved in is model are still relatively unclear, however Ang II is considered to play a key role in the development of the 2K1C model, in which the RAAS system contributes to the development of this renovascular hypertension (Deng *et al.* 2003).

In terms of blocking CGRP activity and function in experimental models, it has been shown that blocking the functional CGRP receptor with $CGRP_{8–37}$ results in an exaggerated cardiovascular inflammatory response due to the onset of hypertension. These include salt-induced hypertension (Supowit et al., 1997; Bowers *et al.* 2005 and Wang *et al.* 2006) and L-NAME-induced hypertension models (Gangula *et al.,*1997). In a model of subtotal nephrectomy hypertension, hypertensive Sprague-Dawley rats showed an additional elevation in MAP when given $CGRP_{8–37}$ (Supowit *et al.* 2001).

1.21 General Conclusion from Introduction

Based on the literature reviewed, this introduction has highlighted the evidence showing that CGRP is a potent vasodilator, especially in the microvasculature, with potential hypotensive properties. However its activities do not appear to contribute to the physiological regulation of BP under normal conditions. The results from a broad range of rodent models suggest a protective mechanism and role for CGRP against the onset of hypertension and vascular inflammation. In hypertensive humans, circulating plasma CGRP levels are generally reduced, and the basis of research in humans is not useful in elucidating the key mechanisms by which CGRP plays a role in this disease process. The study of agents that release CGRP, or act as CGRP agonists are also at very key early stage, however the TRPV1 agonist Rutacarpine which causes release of CGRP has been shown to be anti-hypertensive in rodent models. Suitable CGRP agonists which are currently available may have undesirable peripheral flushing side effects. With the literature in mind, it is clear that further research is required to establish the mechanisms by which CGRP is protective in the onset of hypertension, which therefore leads to the aims and hypothesis this PhD project.

1.22 General Hypothesis and Aims

We have evidence that sensory nerves have an essential role in maintaining homeostasis. These comprise of C- and Aδ-fibre nerves that contain and release the potent vasodilator CGRP. It is well documented from several studies detailed within this introduction that this peptide is considered to play a positive role in wound healing and protects against ischaemic and other traumas (Brain and Grant, 2004). However whilst the potential role of CGRP in neuro-vascular conditions is well documented, the protective mechanisms are less well understood. This PhD was therefore designed to learn more about these protective mechanisms in a model of cardiovascular dysfunction.

The main objective of this PhD was to learn about the contribution of αCGRP to the pathogenesis of Angiotensin II induced hypertension. It is hypothesised that αCGRP plays a protective role in the onset and development of Angiotensin II induced hypertension. The specific aims of the project are detailed below:

- To determine whether CGRP plays a role in baseline BP measurements using wildtype (WT) and αCGRP (KO) mice.

- Design experiments to identify the role of αCGRP and its receptor components in an Ang II induced model of hypertension using WT and αCGRP KO mice in terms of;
    - BP and vasoactive agents
    - Vascular hypertrophy and inflammation
    - ROS and oxidative stress

CHAPTER 2: METHODS AND MATERIALS

2.1 Animals

Wildtype (WT) and alpha CGRP knockout (αCGRP KO) C57BL/6 mice were bred in-house and used in all studies. All were maintained on a normal diet, with free access to food and water, in a climatically controlled environment, on a 12 hour light (7am-7pm) / dark (7pm-7am) cycle. KO mice were age and sex matched with their respective wildtypes. All experiments were carried out in accordance with the Animals (Scientific Procedures) Act 1986. Both genders of mice (18-30g) were used in these experiments at the age of 2 months.

2.2 Generation of αCGRP KO mice

αCGRP KO and WT mice were a gift from Dr. A.M. Salmon, Institute Pasteur, Paris, France. KO mice were created by disruption of exon 5 (specific to αCGRP) of the calcitonin/αCGRP gene by a cassette containing lacz/CMV/neomycin resistance genes (Salmon *et al.* 1999). They were bred from pairs of both heterozygous mice and knockout mice, generating an equal mixture of heterozygous and homozygous knockout offspring. This strain display normal growth and behavioural characteristics, and have been used previously to demonstrate antinociceptive behaviour challenged by morphine (Salmon *et al.* 1999) and to examine BP responses after exercise and phenylephrine infusion (Lu *et al.* 1999). More recently, our group have used these mice to measure vasodilation responses to mustard oil (Grant *et al.* 2005), and to identify any role for αCGRP in the ageing mouse (King, Smillie and Brain, unpublished).

2.3 Genotyping of αCGRP KO mice

Confirmation of the genotype of the mice was carried out using end point polymerase chain reaction (PCR). This identifies the presence (wildtypes/heterozygotes) or absence (knockouts) of the functional αCGRP gene (Figure 2.1). A small ear sample was taken using a simple hole punch method, under 2% isoflurane and 2% O_2 (Abbott Laboratories, Kent, UK) anaesthesia.

Isolation of genomic DNA was done using a commercially available kit (ExtractRED-N-Amp tissue PCR kit, Sigma, UK) according to manufacturer's instructions. The resulting isolated genomic DNA was then added to the PCR mastermix also supplied in the same kit and then amplification of the gene of interest was carried out in a thermocycler (Techne Progrene Thermocycler, Prior Laboratory Supplies, UK, cycle programmes shown in table 2.1) using the following primers for αCGRP:

Primer A: CCCCTAATGGCCTTGTGATTG
Primer B: ACCTCCTGATCTGCTCAGCAG
Primer D: GATGGGCGCATCGTAACCCGT

TEMPERATURE (ºC)	TIME (min)	PROCESS
94	5	Denaturation of DNA and activation of polymerase
94	5	Denaturing of DNA
56	1	Annealing of primers
72	2	Elongation
	(30 cycles)	
72	7	Final elongation

Table 2.1. Thermocycling conditions for the determination of the CGRP genotype by endpoint PCR. Thermocycler programme used to amplify the fragment of the tagged CGRP gene.

The amplified signal was then visualised by gel electrophoresis on a 1.8%, 0.5X tris-borate Ethylenediaminetetraacetic acid (EDTA) (TBE, Biorad) agarose gel in an electrophoresis tank. A DNA ladder (7µl, Bluescript II SK + HPA II digest, Sigma) was used to mark standard base pair sizes, and then subsequent lanes were loaded with 8µl of the genomic DNA product, mixed with 2µl of loading buffer (0.1% w/v Bromphenol blue, 15% w/v Ficoll in running buffer, Sigma). The gel was then run at 90V for 30 minutes which gave sufficient time for the DNA fragments

to separate according to the number of base pairs (bp) present. DNA bands were then visualised under ultraviolet (UV) light (Syngene, G-box).

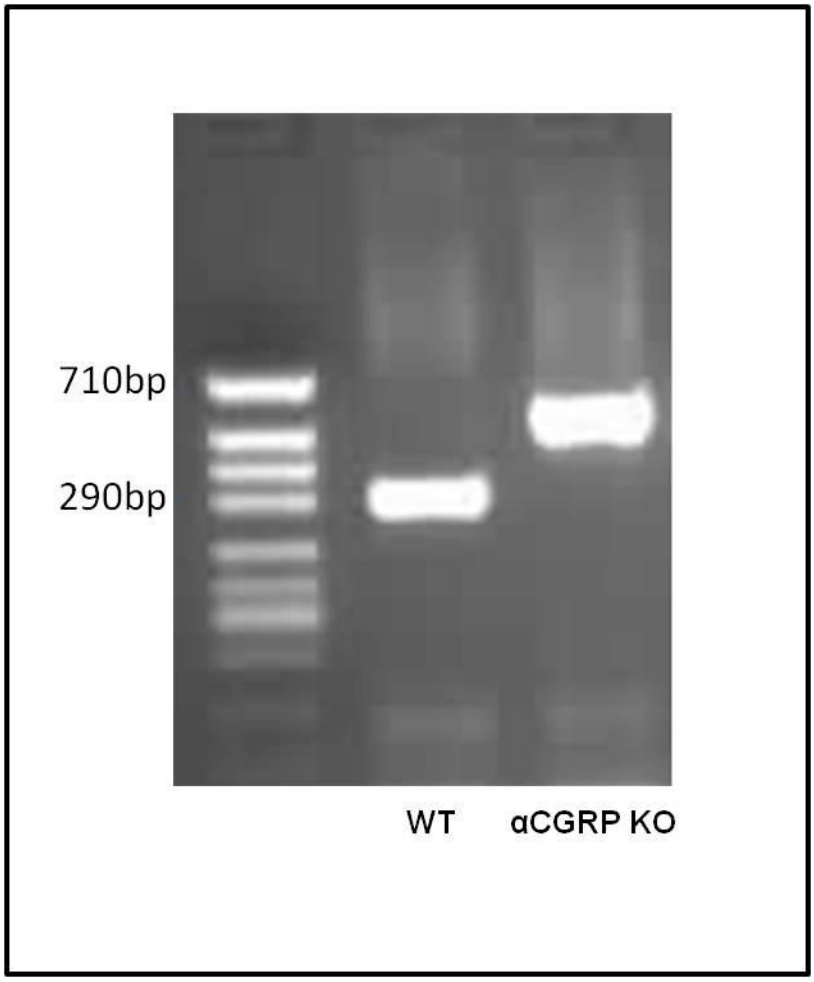


Figure 2.1. Distinguishing αCGRP KO mice from WT mice. Typical gel electrophoresis results showing the products of PCR amplification of the αCGRP gene using genomic DNA from wildtype and αCGRP KO mice.

2.4 Anaesthesia

For the majority of studies, no anaesthesia was necessary. However when it was used e.g. during minipumps implantation, mice were anaesthetised with the inhalation anaesthetic isoflurane (Abbott Laboratories, UK). 4% isoflurane (O_2 carrier gas, 4L/min flow rate) was used to induce anaesthesia and 2% (O_2 carrier gas, 2L/min flow rate) was used for maintenance. During surgical procedures, a deep level of anaesthesia was maintained which was assessed by the loss of paw or tail pinch reflex. After induction of experimental injury the animals were individually housed and allowed to recover in a clean home cage under infra-red light.

Anaesthesia for non-recovery procedures was induced using 4% isoflurane (O_2 carrier gas, 4L/min flow rate) until deeply anaesthetised, followed by cervical dislocation. A deep, surgical level of anaesthesia was maintained throughout all procedures, and was assessed by the absence of a flinch response to pinching of the paw or touching of the eye.

2.5 Angiotensin II Murine Model

Alzet osmotic minipumps (Charles River UK), with either a 14 or 28 day capacity, average volume of 100µl, and an infusion rate of 0.23µl/hr for 14 days or 0.10µl/hr for 28 days, were filled with either saline (vehicle) or angiotensin II (Sigma, UK) at a concentration adjusted to the weight of the mouse. This was to allow for a continuous dose of 1.1mg/kg/day for 14 days (as previously demonstrated by Liang *et al.* 2009) or 0.9mg/kg/day for 28 days. Pumps were implanted subcutaneously in the mid-scapular region via a small incision on the nape of the neck. Implantation was carried out under isoflurane anaesthesia whilst strictly adhering to a surgical aseptic technique. Pain relief was administered intramuscular immediately following surgery with Buprenorphine (50µg/kg, Vetergesic, Alstoe animal health). Insertion wounds were sutured with absorbable sutures (4.0, Ethicon, Johnson and Johnson) in a discontinuous pattern. Mice were then housed singly and monitored daily for signs of pain and infection.

The animals were divided into four groups: WT vehicle (saline), KO vehicle (saline), WT Ang II and KO Ang II. BP was then recorded every second day by tail

cuff plethysmography until day 14 or 28 when the experiment was terminated. This was initiated by anaesthetising the animal with isoflurane until a deep surgical level was maintained, and then blood was collected via the left ventricle of the heart by cardiac puncture. The animal was then sacrificed via cervical dislocation of the neck. Organs were then harvested for post-analysis.

2.6 Measurement of BP via Tail-cuff Plethysmography

As already mentioned in the introduction, there are two main methods of measuring BP in conscious mice, these being tail-cuff plethysmography and radiotelemetry. The advantages and disadvantages of both methods have already been discussed in Chapter 1. In this particular model for this thesis, it was decided that tail-cuff would be the most suitable method for BP measurement. This is primarily because the radiotelemetry technique had not been established within our group when these experiments began. Also, as a personal opinion, because the animals had already undergone surgery for the osmotic minipump implant, the extent of an additional radiotelemetry implant could therefore have limited the rate of survival. In this particular case with the tail-cuff technique, we had a survival rate of 99%, which also fits in with the 3Rs and kept our experimental costs to a minimum.

BP was measured by tail cuff plethysmography (as previously described by Clark *et al.* 2007 and Tam *et al.* 2006), using the CODA 6 non-invasive BP acquisition technique system for mice (Kent Scientific, Torrington, CT, USA). This is a non-invasive computerised system uses volume-pressure recording to measure BP and has been successfully used previously (Feng *et al.* 2008; Bunag, 1973; Mahoney and Brody, 1978; Liang *et al.* 2009). This system has been validated by the company against telemetered animals. Mice were restrained in purpose designed humane restraining tubes. An occlusion cuff was placed at the base of the tail which occluded blood flow into the tail before being softly deflated. A second cuff (volume pressure recording, VPR) was placed at the mid way point of the tail and was used to measure the change in volume resulting from the returning blood flow. As blood returns to the tail, it swells (measured as systolic pressure). Once the tail has ceased swelling, diastolic pressure and MAP are calculated based on the systolic value. This was then recorded as a wave form in

the CODA 6 data acquisition software and numerical values were calculated from this. The first five measurements were disregarded as the mouse had to acclimatise to the sensation of the measurement due to the cuffs. Thereafter, the mean of at least five BP measurements were taken per mouse each day. All measurements were taken at a thermoneutral ambient room temperature of 25-27°C. Mice were also warmed on a heating pad for 10 minutes prior and during the BP recordings. This ensured that the tail blood vessels were not constricted for thermoregulation purposes, but relaxed, allowing blood flow changes to be reliably observed. Mice were trained for at least 14 consecutive days prior to taking the BP measurements in order to reduce stress-induced hypertension caused by restraint.

2.7 Termination of experimental hypertension

At day 14 or 28 after implantation, final BP measurements were taken and then the mice were terminated. This was done by induction of anaesthesia and then cervical dislocation. Prior to death, blood was collected via cardiac puncture. Final body weight was recorded and mice dissected so that the heart, aorta, kidneys, dorsal root ganglia (DRG) and mesenteric resistance vessels (MRV) could be stored for future work.

2.7.1 Markers of Cardiovascular Disease

Following termination of the animal at days 14 or 28, organs were dissected and weighed. Placing the mouse abdominal side up, the thorax was opened by cutting at the sternum and through the ribs. Firstly, the heart was removed by snipping the top of the aortic arch, the inferior and superior vena cava and the pulmonary artery and vein. After being immersed in ice cold saline to remove any blood, the heart was blotted dry and trimmed of any remaining vessels before being weighed whole. The left ventricle was located and gently pinched away from the rest of the heart wall to feel the ventricular space, and then cut free before being weighed. Following from this, in the same manner, the right ventricle was located on the opposite side. The procedure to remove and weigh this is as described above. The lungs were then located and cut free from the chest cavity. This then allowed

the thoracic aorta to be in view for removal. The descending thoracic aorta is attached to the interior side of the spine by connective tissue. Just below the thyroid, the aorta was removed by carefully cutting away in a downwards direction towards the abdomen. Care was taken to avoid any damage by stretching or cutting. After cutting down to the level of the abdomen, the aorta was cut free and washed gently in ice cold saline to remove any blood. After weighing, all samples were snap frozen in liquid nitrogen and stored at -80°C until needed. Any samples being used for molecular techniques were immersed in RNALater (Invitrogen, UK) before being snap frozen and stored at -80°C until needed. Samples for histology were fixed in formaldehyde (Sigma, UK) and stored at room temperature.

2.7.2 Other markers of Disease

Following dissection of thoracic organs, the abdominal wall was cut open to expose the abdominal organs. Any excessive abdominal fat was removed in order for the organs to be seen clearly. The intestines, caecum and stomach were removed intact and mesenteric fat and the mesenteric artery were dissected before disposing of the remaining tissue. This was cleaned and prepared for post analysis. The liver was then removed by holding the liver at the points where each individual lobe joins to the others and cut free from the abdomen with care in order to not damage the delicate tissue. This was washed and blotted dry before being weighed. Both right and left kidneys were then removed and carefully excised from their protective renal capsule before being weighed. After trimming away the renal artery and vein, the kidneys were then washed, blotted dry and weighed. After weighing, all samples were snap frozen in liquid nitrogen and stored at -80°C until needed. Any samples being used for molecular techniques were immersed in RNA later (Invitrogen, UK) before being snap frozen. Samples for histology were fixed in formaldehyde (Sigma) and stored at room temperature.

The DRG was collected from the dissection of the spine and then stored in RNA later (Invitrogen) at -80°C until needed for molecular use.

The spleen was dissected and weighed in order to find any potential abnormalities in animals such as microbiological infection which may affect any results. This was also the same for the lungs and liver. Any unusual observations (e.g. renal

cysts) were recorded and mice with any significant abnormalities were removed from the study.

2.8 Echocardiography

Echocardiography was performed by Dr James Clarke (Kings College London) using a VEVO 770 with a mouse scan head (RMV 707B, 30HZ, VisualSonics, Inc., Toronto, Canada). It is thought that this technique can measure parameters such as left ventricular (LV) mass as accurately as when collected during gross dissection; therefore it is a common and popular technique for the assessment of genetically modified mouse phenotypes and for monitoring heart disease progression. A detailed description of how the echocardiography is carried out is described in the materials and methods section of the published online video (Respress and Wehrens, 2010).

Three main sections were collected for analysis as displayed in figure 2.2. a) pasternal long axis- where the LV apex is at maximum length and aortic root is in full view. Measurements were collected at the level of the papillary muscle for consistently comparable cross-sections. b) short axis view with another shot between the two papillary muscles. These images were then analysed using the leading edge method over a period of 5 sinus beats. Calculations were then made immediately by the integrated software. The last image is c) an aortic wave Doppler which was used to observe systolic function. Mouse weight was recorded at the beginning of the procedure and measurements are expressed as a ratio to body weight. Morphological measures which were taken include LV Mass which measure the whole calculated mass of the left ventricle and is the ultimate measure of heart tissue size. Heart mass was also determined from this and expressed as a ratio to body weight. Functional measurements were also calculated including stroke volume; the measurement of the amount of blood ejected from the heart each beat and a functional measurement of heart output. Ejection fraction indicates the percentage of blood ejected from the left ventricle and finally cardiac output which is the most commonly used measurement of cardiac output, combining the stroke volume with the heart rate.

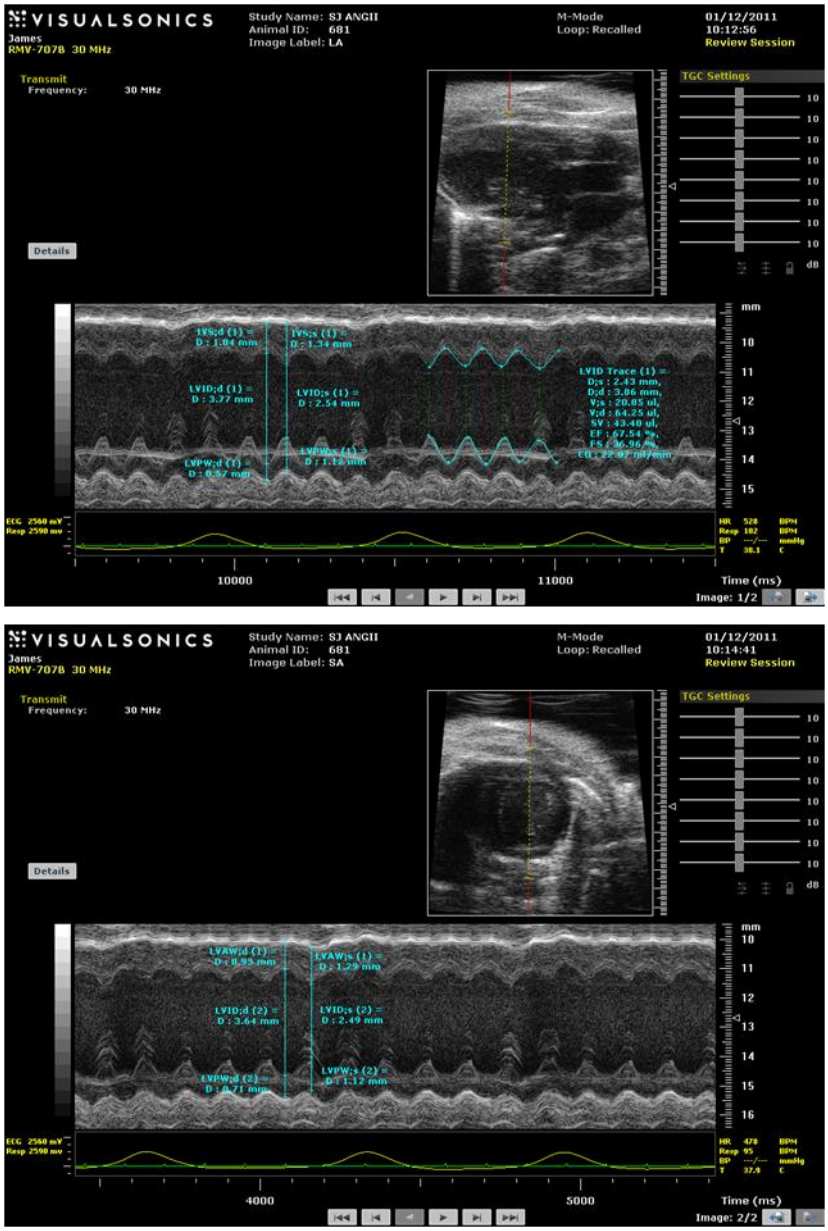


Figure 2.2. Echocardiography sample measurements. Views of measurements from long axis (top) and short axis (bottom).

2.9 Plasma analysis

2.9.1 Preparation of plasma samples

As mentioned previously, whilst under terminal anaesthesia, a blood sample (1ml) was obtained via a cardiac puncture through the left ventricular wall using a 25G needle and 1ml syringe previously coated with heparin (500 I.U/ml heparin sodium) in order to prevent coagulation of the blood. This was then transferred into a 1.5ml micro centrifuge tube and centrifuged at 2000rpm for 10 minutes at room temperature. The supernatant was transferred into a new micro centrifuge tube and snap frozen in liquid nitrogen before being stored at -80°C until further use.

2.9.2 Endothelin 1 (ET-1) enzyme-linked immunoassay (ELISA)

Plasma samples from each animal were assayed for ET-1 detection by ELISA, an *in vitro* test which uses the quantitative sandwich enzyme immunoassay technique. All reagents were obtained from Phoenix Pharmaceuticals Inc, USA. Plasma samples had to be purified first in order to allow the extraction of peptides using SEP-columns (Phoenix Pharmaceuticals Inc, USA) according to manufacturer's instructions and then freeze drying the purified sample overnight before beginning the assay. The ELISA involved an immunoplate pre-coated with secondary antibody to ET-1 with non-specific binding sites blocked. The secondary antibody can bind to the Fc fragment of the primary antibody (ET-1 peptide antibody) whose Fab fragment will be competitively bound by both biotinylated peptide and peptide standard or targeted peptide in samples. The biotinylated peptide interacts with streptavidin-horseradish peroxidise (SA-HRP) which catalyses the substrate solution. The intensity of the yellow colour formed is directly proportional to the amount of peptide in standard solutions or samples. This is due to the competitive binding of the antibody. A standard curve of known concentration was established accordingly and the unknown concentration in samples was determined by extrapolation to this standard curve and expressed as pg/ml.

2.9.3 Measurement of pro-inflammatory cytokines- Cytokine MSD Multiplex ELISA

Plasma cytokines IL-1, IL-6, KC (mouse IL-8) IL-10, IL-12 and TNFα were evaluated using a pro-inflammatory multi-plex cytokine kit (Mouse Pro-Inflammatory multi-plex kit, Meso-Scale Discovery, Gaithersburg, MD, USA) according to manufacturer's instructions and using reagents supplied by the manufacturer. MSD cytokine assays measure 1-10 cytokines in a 96-well multi-array or multi-spot plate. The assay employs a sandwich immunoassay format where capture antibodies are pre-coated in a single spot, or in a patterned array, on the bottom of each wells of the plate. When the plasma sample was incubated on the plate, the cytokine in question was able to bind to the appropriate antibody capture spot. 25μl of mouse serum diluents was added to each well and then the plate was incubated for 30 minutes at room temperature, whilst constantly shaking at 200rpm. Following on from this, the standards and plasma samples were added to the wells and then left to incubate for a further 2 hours, again whilst shaking at 200rpm. At the end of the 2 hours the plate was washed 3 times with 0.05% PBS Tween-20 (Sigma). At this point the labelled cytokine-specific antibody was added (25μl) to each well and then incubated for a further 2 hours at room temperature, shaking at 200rpm. Finally the reading buffer was added (150μl) to each well and then the plate was analysed by a SECTOR instrument SI6000 plate reader (Meso Scale Discovery, Maryland, USA). Sample readings for each cytokine were compared with a supplied standard curve (0-10,000 pg/ml) and results were expressed as cytokine levels in pg/ml. All detection antibodies exhibited less than 1% cross-reactivity with other analytes.

2.9.4 Total plasma CGRP (α and β isoforms)

Circulating plasma CGRP levels were measured using a Rat/ Mouse ELISA kit from Phoenix Pharmaceuticals Inc, USA, all reagents included). This kit detects 0.16-100ng/ml of CGRP. This kit measured both α and β isoforms of CGRP in the plasma. Plasma peptides were extracted using SEP-COLUMN's (Phoenix Pharmaceuticals Inc, USA, procedure carried out in accordance to manufacturer's instructions) prior to the ELISA to ensure samples were concentrated and purified. In the assay, the plate was pre-coated with secondary antibodies to the primary

CGRP antibody. This antibody was competitively bound by CGRP in the sample/standard or by biotinylated peptides, and when bound, a yellow coloured substrate was produced in which the colour intensity was directly proportional to the amount of CGRP bound to the primary antibody, therefore the concentration of CGRP found in the sample could be measured. Results were expressed as pg/ml and normalised to plasma protein levels. Cross-reactivity occurs with all CGRP forms (78.6% with CGRPβ rat and 20.1% human) and to a certain degree with CGRP from other species (100% with rat and 15.1% with human). However it is not cross-reactive with closely related peptides such as calcitonin (0%) and amylin (<0.01%).

2.9.5 Plasma αCGRP ELISA

This assay was carried out under the exact same principles as described above, however the ELISA assay was obtained from Peninsula Laboratories (part of the Bachem group), USA. This assay detected 0-10ng/ml of αCGRP and had 1% cross reactivity with closely related peptides (amylin and calcitonin), and 23% cross reactivity with the βCGRP isoform.

2.10 Histology

2.10.1 Processing and cutting tissue (Aorta) for histological analysis

Fixation

Prior to staining, tissue was fixed in 10% paraformaldehyde (Sigma) for 24 hours to prevent autolysis and necrosis, and preserve the antigenicity of the tissue.

Processing and Paraffin-Embedding

After fixation, processing was completed using an automatic tissue processor (Tissue-Tek VIP Vacuum Infiltration processor, Sakura Tissue Tek VIP 1000). Tissues were dehydrated using graded alcohols (100%, 90% and 70%), cleared

with xylene (100%), and infiltrated with paraffin wax. The tissue was subsequently embedded with paraffin wax in moulds or cassettes, using a Sakura Tissue Tek Embedding Centre, which facilitate tissue sectioning and kept at room temperature.

Sectioning, tissue preparation, removal of paraffin and dehydration

Tissue was then cut in 5 micron transverse sections using a microtome (Reichert-Jung 2030 Biocut microtome). Ribbons of cut tissue were floated onto water heated to 40°C to smooth out any creases in the tissue and then picked up onto polylysine coated slides (Sigma-Aldritch, Poole, Dorset, UK). Ten slides were mounted with 3 to 4 sections on each slide to give a good representation of each individual subjects aortic profile. To enable sufficient fixing of the tissue sections to the slides, they were dried and baked in an incubator at 45°C overnight. At this point the tissue was then ready to be deparaffinised. This procedure was carried out with caution as any residual embedding media resulted in increased nonspecific or reduced staining. This involved a simple protocol of immersing the slides in xylene (VWR BDH Prolab International Ltd) for 10 mins, followed by 5 minutes in 100% ethanol (VWR BDH Prolab International Ltd), 5 minutes in 95% ethanol and then 5 minutes in 70% ethanol (Sigma). Slides were then immersed in running distilled water for 5 minutes. The deparaffinised slides slides were then ready for any staining procedure.

2.10.2 Masson's Trichrome stain

Masson's Trichrome is a three colour staining protocol commonly used in histology to distinguish cells from surrounding connective tissue. In this case, it was used to distinguish the surrounding collagen from the smooth muscle layer of the aorta. Red staining occurs in keratin and muscle fibres, blue indicates collagen, pink identifies the cytoplasm and the cell nuclei are stained dark brown/black. Pre-cut sections of the thoracic aorta, sectioned at 5µm intervals from paraffin blocks, were firstly deparaffinised as described previously. Slides were then immersed in 65ºC Bouin's solution (Sigma) before being immersed in a series of ready- to- pour stains, in the order of haematoxylin, Biebrich Scarlet solution (0.9% Biebrich scarlet, 0.1% fuchsin and 1% acetic acid, Sigma) and

Analine blue (2.4% analine blue, 2% acetic acid, Sigma) as per manufacturer's instructions. The resulting stained sections were then dehydrated through graded ethanol (100%, 90% and 70%) and immersed in xylene (100%) before being dried and mounted and coverslipped with pertex xylene based mountant (Sigma). Finished slides were then left to dry in the fume cupboard overnight before any analysis was conducted. Analysis of this staining procedure is described in Aortic Imaging.

2.10.3 Immunohistochemistry (IHC): VCAM-1, CGRP and NOX4

Immunohistochemistry (IHC) is the process of localising proteins in cells of a tissue section by the principle of antibodies binding specifically to antigens (Elias, 2003). Specific molecular markers are characteristic of particular cellular events such as proliferation or apoptosis. IHC is used in research to understand both distribution and localization of biomarkers and differentially expressed proteins in different parts of a tissue (Ramos-Vara, 2005). The fundamental concept behind IHC is the detection of antigens (Ag) within tissue sections by means of specific antibodies (Abs) (Absolom and Van Oss, 1986). Once antigen-antibody (Ag-Ab) binding occurs, it is revealed with a coloured histochemical reaction visible under the microscope. Visualising an antibody-antigen interaction can be accomplished in a number of ways. Most commonly, an antibody is conjugated to an enzyme, such as peroxidase, that can catalyse a colour-producing reaction (Adams, 1992).

The method of immunohistochemical staining, which is used in this particular study, uses an antibody raised against the antigen being probed for, and a second labelled, antibody against the primary antibody. A biotinylated secondary antibody is then coupled with streptavidin horseradish peroxidase. This is reacted with 3,3'-Diaminobenzidine (DAB) to produce a brown staining wherever primary and secondary antibodies are attached in a process known as DAB staining.

<u>The protocol</u>

Formaldehyde is known to induce conformational changes in the antigen molecules by forming intermolecular cross-linkages. Excessive formalin fixation can mask antigenic sites and diminish specific staining. However, these sites may be revealed with proteolytic digestion or target retrieval of tissue slides prior to

immunostaining. In this case, once the slides were deparaffinised as described previously, we used heat induced epitope retrieval, results in increased staining intensity with many primary antibodies. In this thesis we used citrate tris buffer at pH 6.0 (Vector Labs) in a container and microwaved at full heat for 5minutes. Other ways of inducing heat include a water bath, steamer or pressure cooker.

After antigen retrieval, slides were washed in running tap water followed by a short immersion in PBS-Tween-20 (5mins) before being blocked in 3% hydrogen peroxide (Dako) for 30 minutes. This step was used to block any unspecific background staining which may occur due to unspecific binding of the primary antibody, which therefore gives a cleaner end result. This blocking step was stopped by a 5 minute wash in distilled water. Sections were then blocked in 3% normal blocking serum (Dako). The preferred serum for blocking is from the same species in which the biotinylated secondary antibody is made. In this case it was rabbit serum (Dako). After 30 minutes of blocking, the primary antibody (CGRP, NOX4 and VCAM-1) diluted in Dako REAL antibody diluent at a concentration of 1:500 was applied and left overnight at 4°C. All antibody used were polyclonal and raised in rabbit. The anti-CGRP (total, α and β) antibody was a gift from Catia Sternini, Professor of Medicine and Neurobiology David Geffen School of Medicine, UCLA, previously utilised and published (Cottrell *et al.* 2005). The anti-NOX4 antibody was a gift from Prof Ajay Shah, Kings College London, London (Schroder *et al.* 2012). Anti-VCAM-1 (Ab106777) was a commercial antibody obtained from Abcam, UK (Liang *et al.* 2009). A negative control was also used in all staining procedures for this study to verify the specificity of the labelling of the target antigen by the primary antibody. The absence of specific staining in the negative control tissue confirmed the lack of antibody cross-reactivity to cells/cellular components. If specific staining had occurred in the negative control, results were considered invalid. In negative tissues, the tissues had a blue-purple appearance when using haematoxylin. Non specific staining, if present, had a diffuse appearance.

Following overnight incubation, slides were washed for 3x5 minutes in PBS-Tween20 at room temperature on a shaker. Slides were then incubated with secondary antibody (mouse-anti-rabbit, Dako) for 30 minutes at room temperature. This was followed by 3x 5 minute washes on a shaker with PBS-Tween.

DAB (Vector Labs) was dropped onto sections and left to react until the sections were visibly brown (roughly 6-8 minutes). To stop the reaction the slides were immersed in running tap water for 5 minutes. In order to counterstain the sections so they would be visible for examination under the microscope, slides were stained with Harris' haematoxylin (Sigma) for 1 minute followed by washing in running tap water for a further 5 mins to wash away any excess stain. Slides were then rehydrated through a series of ethanol and xylene (as described previously) before being mounted with pertex xylene based mountant (Sigma) and coverslipped.

2.10.4 Aorta Imaging

Aorta sections were imaged using a colour video camera (Olympus U-CAMD 3, soft imaging system), connected to a calibrated microscope (Olympus BX51). Images of aortic rings were measured using specialised morphometry software, (Cell P Software © 1986-2007, Imaging Solutions, GmbH). After Masson's trichrome staining, aortic wall width was measured under 40x magnification and aorta area was measured under 20x magnification. The best and most representative 4-5 sections were selected for analysis. Aortic wall width was calculated by taking 8 measurements per section at approximately 45 degree angles. As 4-5 sections were measured per mouse, aortic wall width measurements are therefore the mean of 32-40 measurements per animal. Aortic area was calculated by measuring the circumference of the inner lumen and subtracting this from the circumference of the outer edge of the aorta. Aortic area measurements were a mean of 4-5 measurements per mouse. Immunohistochemistry for VCAM-1 was measured by free-hand drawing around areas of positive staining for VCAM-1 and measuring it as a positive area in % of total area. This again was measured on 4-5 measurements per mouse. NOX4 and CGRP staining were measured by densitometry. This procedure involved measuring how dense the positive staining was. We excluded any light brown staining as this was probably excessive background staining. Measurements for dark brown staining were considered as positive staining and collected. Results are expressed as % of positive staining relative to total aorta area. Densitometry was calculated on a mean of 10 measurements per mouse, and was further

divided into 3 layers; endothelial, smooth muscle cell and collagen/adventitia. This was to allow for localisation of the protein to be determined. All histology samples were analysed blind and referred to by an identifying reference number only. All morphological analysis (aortic width and area) was performed blind by two individuals and the mean of the two measurements was taken as the final result.

2.11 Measurement of Gene Expression using Real-Time Polymerase Chain Reaction (RT-PCR)

2.11.1 RNA Isolation

Unlike DNA, RNA is very easily and rapidly degraded by RNases. Autoclaving glassware and solutions is not enough to inactivate all RNases. Even when using RNase-free materials, you can easily introduce RNases unless great care is taken. Gloves were worn at all times when handling RNA, and all materials used in RNA isolation were RNase-free. Commercial kits and reagents specifically for RNA isolation were certified nuclease-free, and any other materials and work surfaces used in these experiments whilst handling RNA were made RNase-free using RNAse ZAP (a RNA decontamination solution, Invitrogen, UK). Nuclease-free filtered disposable pipette tips were used for all procedures in this study and were obtained from Starlab, UK.

2.11.2 RNA extraction- Spin column method

Small (10-30mg) tissue samples (heart, kidney, aorta, mesenteric resistance vessels and dorsal root ganglia) were quickly excised from the animal and immersed in RNAlater (Ambion) in a nuclease-free 1.5ml micro centrifuge tube tube and left overnight at 4^0C. Tissue was then stored at -80^0C until required.

Total RNA was extracted from the tissue using the Qiagen RNeasy Microarray spin column kits (Qiagen) according to manufacturer's instructions. This kit provides all the materials required to isolate total RNA of >200 nucleotides using spin columns and their patented phenol-guanidine-based QIAzol Lysis Reagent. Briefly, the procedure involves the rapid lysis of tissue in order to quickly inactivate any cellular RNases and protect sensitive RNA from degradation. To do this, 10-30mg of tissue was placed into 1ml of QIAzol in a 2ml round bottomed micro centrifuge tube with a 5mm stainless steel bead (Qiagen). The tissue was then disrupted using the Qiagen TissueLyser® LT. Lysis was carried out for 2x 2 minutes at 20 Hz, or until the tissue was completely dispersed. The homogenate was then treated with chloroform (Sigma) before being centrifuged, and resulting

precipitated RNA was sequentially purified and eluted using the spin column procedure. This procedure involves loading the sample onto the spin column and spinning and washing with the supplied buffer a number of times. During these steps, RNA is absorbed on the silica gel membrane of the spin column, whereas unwanted cellular contents (protein, DNA) are washed away. The total RNA was then eluted in nucleotide-free water and RNA concentration was measured on a Nanodrop spectrophotometer.

2.11.3 Measurement of RNA quality

RNA analysis on the NanoDrop:

Determination of RNA quality was done by measuring absorbance at 260nm (A_{260}) in a Nanodrop spectrophotometer. An absorbance of 1 unit at 260nm corresponds to 40µg of RNA/ml. The ratio of A_{260}/ A_{280} provides a measure of RNA purity with respect to contaminants such as protein (which absorbs at 280nm). In addition to this, the A_{260}/A_{230} ration was measured as an indicator for ethanol and phenol contamination (which absorbs at 230nm). A ratio close to 2 (1.8-2.2) suggests good purity. There is no method available which can guarantee that the RNA is completely free of contaminating genomic DNA. Spectophotometry (in this case the NanoDrop) does not give any information about the quality of the RNA that is isolated. Heavily degraded RNA and completely intact RNA will both give a peak at 260nm and may have a good 260/280nm ratio, therefore determination of RNA integrity was carried out next.

RNA analysis on the Agilent 2100 Bioanalyzer:

RNA samples were analysed using the RNA nano chip as per manufacturer's instructions (Applied Biosystems). 1ul of RNA sample was premixed with the Agilent mastermix and loading dye supplied with the kit before being loaded into the well of the chip. The chip is then loaded onto the Agilent 2100 to be bioanalysed. This instrument is designed for sizing and analysis of RNA, DNA or protein using small samples which are rapidly separated through very fine

microchannels in specific RNA, DNA or protein 'chips'. The RNA Nano chip has a series of interconnected microchannels that are used to separate nucleic acid fragments based on their size as they are driven through it electrophoretically. The resulting electropherogram is a very good tool in assessing the RNA quality. Generally, the presence of two prominent ribosomal peaks (18S and 28S) and 1 marker peak indicates successful sample preparation. Another good feature of good quality RNA is a flat baseline.

If more peaks are visible with a higher background at a lower molecular weight than the 18S band, this suggests poor sample quality and likely RNA degradation. 18S/28S ribosomal ratios may vary according to the species and tissue type as well according to the RNA extraction method.

The Bioanalyzer assigns a RNA integrity Number (RIN) to each sample run; 10 is perfect RNA (i.e. no evidence of any degradation) and lower numbers indicate progressive degradation. In using RT-qPCR to compare samples it's important that all have a similar (preferably small) degree of degradation (i.e. a similar RIN). Samples with a RIN lower than 6 were omitted from this study.

2.11.4 Reverse Transcription

In order to perform RT- qPCR, isolated RNA was first reverse transcribed into cDNA using the High Capacity RNA-to-cDNA Kit from Applied Biosystems, as per manufacturer's instructions. This kit provides a fast and convenient procedure for efficient reverse transcription (RT) resulting in a high yield of cDNA, even from low abundance RNAs. In this study, 500ng of extracted RNA was used for the reverse transcription stage. 500ng of RNA was added to 10µl of RT mix and then made up to 20µl with RNA-free water in a 0.2ml RNAse-free micro centrifuge tube. RT was then run using a thermal cycler (Applied Biosystems) which was pre-set according to the manufacturer guidelines. The cycle was as follows; 37ºC for 60mins, 95ºC for 5 mins and then cool and hold at 4ºC for 5minutes. Following the RT step, stock concentrated cDNA was then diluted 1:5 for RT-qPCR.

2.11.5 RT-qPCR

Real-time PCR was performed using a SyberGreen based PCR mix (Sensi-Mix®, SYBR-green no ROX, Bioline.) and primers from the specific gene of interest. A list of primers used is shown in Table 2.2 and were all obtained from Sigma, UK. A 10µl reaction mix was then made up of the components illustrated in Table 2.3. Once this mix was prepared, samples were added to 100 well gene discs (Qiagen) using an automated robot (CAS1200, Corbett Robotics). Loaded discs were then run for PCR amplification in a Corbett Rotorgene which determines copy numbers via fluorescence of the SYBR-green using the following cycle; hold for 10mins at 95^0C, cycling for 45 cycles- 10 sec at 95^0C, 15 sec at 57^0C, 5 sec at 72^0C and finally melt 68-90^0C. Raw data was analysed using the Rotorgene 6000 series software and expressed as copies/µl. Quality control and efficiency of the PCR reaction was assessed by performing a melting point analysis after each run. Melting point analysis is used to distinguish target amplicons from PCR artefacts such as primer dimers or misprimed products. In order to compare gene expression between samples, it was necessary to normalise the qPCR data to account for variations in starting material, total or mRNA extraction and differences in reverse transcription efficiency between samples and individuals. Normalisation in this case was done running endogenous reference genes, also known as “housekeeping” genes which are known to be abundant in all samples and do not vary with experimental treatments. In this study, reference genes used were hypoxanthine guanine phosphoribosyl transferase (HPRT-1), succinate dehydrogenase (SDHA) and phospholipase A_2 (PLA_2). These reference genes were selected as they are expressed at a constant level across various conditions, and their expression is assumed to be unaffected by experimental parameters such as Ang II and in models of hypertension (Zhaoguo *et al.* 2009). The ratio of gene of interest to reference gene is then the measure used to compare samples. It is advised to normalise genes by using the geometric mean of several reference genes, (in this case we used three). To calculate the geometric mean of the reference genes we used the GeNorm version 3.4 software. Final results are expressed as copy number per µL of pure cDNA normalised by comparison to HPRT-1, SDHA and PLA_2. All experiments were performed in accordance with the MIQE guidelines. It is advised to perform RT-qPCR on all samples at the same time in order to keep all experimental conditions

tightly regulated in order for a direct comparison to be made. This minimises experimental errors such as standard degradation, variations in sample preparation and the efficiency of the reverse transcription and PCR amplification. However in this study, the results obtained from the 14 day Ang II study were the basis for the idea of performing a 28 day Ang II study, therefore gene expression was not measured at once for both of these studies. In order to minimise any experimental variation and to give an accurate comparison, results were expressed as a fold change from the expression displayed by the vehicle treated animals. This then allowed us to compare the trends from each study. These results are discussed in Chapter 7.

	Forward Sequence	Reverse Sequence	Amplicon Size
CGRP α	AGCAGGAGGAAGAGCAGGA	CAGATTCCCACACCGCTTAG	71
CGRP β	CCTGCAGGCCTGAGTCAC	GGCATGGTGAGTTCAACTTTATG	64
CLR	CTCCTGAGACTATTCCCACAGAA	CAAGATGTTGCTGTATCATCATAGG	72
Collagen III	GTTCTAGAGGATGGCTGTACTAAACACA	TTGCCTTGCGTGTTTGATATT	76
eNOS	GACCCTCACCGCTACAACAT	GTCCTGGTGTCCAGATCCAT	62
ET-1	GTGTCTACTTCTGCCACCTGGACAT	GGGCTCGCACTATATAAGGGATGAC	474
GPX	TTTCCCGTGCAATCAGTTC	TCGGACGTACTTGAGGGAAT	76
HO-1	GGTCAGGTGTCCAGAGAAGG	CTTCCAGGGCCGTGTAGATA	70
HPRT1	TCCTCCTCAGACCGCTTTT	CCTGGTTCATCATCGCTAATC	90
ICAM-1	CCCACGCTACCTCTGCTC	GATGGATACCTGAGCATCACC	72
IL-6	GCTACCAAACTGGATATAATCAGGA	CCAGGTAGCTATGGTACTCCAGAA	78
MCP-1	ACTGAAGCCAGCTCTCTCTTCCTC	TTCCTTCTTGGGGTCAGCACAGAC	274
NOX-2	TGCCAACTTCCTCAGCTACA	GTGCACAGCAAAGTGATTGG	73
NOX-4	GAACCCAAGTTCCAAGCTCA	AAGGCACAAAGGTCCAGAAA	63
PLA2	TGGATATAAACCATCTCCACCA	GGGAAGGGATACCTATGTTCAGA	77
RAMP 1	TGACTATGGGACTCTCATCCAG	CGTGCTTGGTGCAGTAAGTG	137
RAMP 2	CTGAGGACAGCCTTGTGTCA	GGCATCGCTGTCTTTACTCC	374
RAMP 3	CCTTCTTCCACTGTTGTTGCT	CTCTCCAGCATCCCTGTCTC	78
SDHA	CCCTGAGCATTGCAGAATC	TCTTCTCCAGCATTTGCCTTA	70
SOD	CTGGACAAACCTGAGCCCTA	GATAGCCTCCAGCAACTCTCC	61
TGF-beta	GGCGGTGCTCGCTTTG	GCTGATCCCGTTGATTTCC	496
TRPV1	CAACAAGAAGGGGCTTACACC	TCTGGAGAATGTAGGCCAAGAC	77
VCAM-1	TGGTGAAATGGAATCTGAACC	CCCAGATGGTGGTTTCCTT	86

Table 2.2 List of genes and their primer sequences and amplicon size (base pairs) used for RT-qPCR. Working solutions were diluted 1 in 10 from the primer stocks. All primers were obtained from Sigma.

Component	µl
Forward Primer	0.4µl
Reverse Primer	0.4 µl
Mastermix (SYBER)	5 µl
H_2O	3.6 µl
cDNA	1µl
Total	10 µl

Table 2.3 Relative components which make up the final mastermix used for RT-qPCR.

2.12 Western Blotting for aortic HO-1 and NOX4 protein expression

2.12.1 Sample preparation

Tissue was washed free of blood using saline and snap-frozen in liquid nitrogen. Samples were stored at -80ºC. Tissue was weighed and homogenised in 10ml per gram weight of tissue homogenising buffer (RIPA buffer, 150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), and 50 mM Tris, pH 8.0. Sigma), containing protease inhibitor cocktail (Sigma). Tissue was then homogenised using a tissue lyser (Qiagen Lysis Cube). A 100ul aliquot of homogenised sample was added to an equal amount of 2X sample buffer, 2-mercaptoethanol (Sigma) added to 10% (v/v) and heated to 95ºC for 5mins. Samples were cooled and 3ul 8% bromophenol blue (in 100% ethanol, Sigma) was added. Prepared samples are stored at -20ºC.

2.12.2 Equalisation of protein loading

A Bradford assay was performed in order to determine protein concentration as per manufacturer's instructions (Thermo scientific).

2.12.3 One-Dimensional (SDS)-Page

Acrylamide gels were poured between two glass plates separated by 1mm spacers (equipment from Biorad). The gels were made of a lower "resolving" gel and an upper "stacking" gel. The resolving gel (pH 8.8) appropriate to the experiment was made from a mixture of a 30% acrylamide solution, Tris-buffered SDS solution (8.8) and ddH_2O to give an acrylamide gel of 12% (All reagents from Biorad or Sigma). Prior to pouring NNNN-tetraethylethalinediamine (TEMED) (Sigma, UK) and ammonium persulphate (APS) (BDH, UK) were added to the mixture to induce acrylamide polymerisation and cross-linking. Immediately after pouring, water-saturated isobutanol (BDH, UK) was layered over the gel mixture to prevent the formation of air bubbles and drying. Following acrylamide

polymerisation the isobutanol was washed off and the upper “stacking” gel (pH 6.8) was prepared by a mixture of 30% acrylamide, Tris-buffered SDS solution (pH 6.8) and ddH_2O to give a 5% mixture. The acrylamide was polymerised by addition of TEMED and APS poured on to the resolving gel; a ten-well well-forming comb (Biorad) was immediately inserted into the gel to form the wells into which the protein samples were loaded. 25-50ug protein was run in the gel alongside 6ul of molecular weight protein marker (Sigma, UK) at 90V in 1X gel running buffer until protein of interest was suitably separated as determined by migration of the dye.

2.12.4 Western Blotting

Western blotting was carried out as follows; briefly, proteins were transferred to Hybond-P nitrocellulose membranes (Biorad) at 100mA for 1 hour using a Biorad Mini-blot apparatus. Membranes were then blocked in PBS/Tween plus 3.0% milk powder (Marvel) for 1 hour. Blots were then probed with primary antibody (HO-1 which was a gift from Dr Richard Siow, or NOX4 which was a gift from Prof Ajay Shah, both of which were raised in mouse) typically at 1:1000 dilution in PBS containing 0.005% Tween-20 and 0.1% milk at 4ºC overnight with constant gentle shaking. HRP-conjugated second antibody was used at 1:2500 dilutions for 3 hours (Dako Rabbit anti-mouse). Blots were developed using an ECL kit (G.E. Healthcare) and exposed to film (Kodak) for between 30 seconds and 2 minutes. All blots were normalised to α-tubulin as a control. To do this, membranes were washed with PBS Tween-20 overnight and then re-probed with α-tubulin (1:1000 dilution in PBS containing 0.005% Tween-20 and 0.1% milk) overnight at 4ºC with constant gentle shaking. HRP-conjugated second antibody was again used at 1:2500 dilutions for 3 hours (Dako Rabbit anti-mouse). Blots were again developed using an ECL kit (G.E. Healthcare) and exposed to film (Kodak) for between 30 seconds and 2 minutes. Visualised bands were analysed using Image J® densitometry software and results were expressed as fold change from the control and normalised to the α-tubulin control. Bands for the gene of interest were detected according to their molecular weight, HO-1 (32kDa), NOX4 (65kDa) and α-tubulin (50kDa).

2.13 Data Analysis

Raw data were analysed in Microsoft Excel and Graphpad Prism 5. All data were expressed as mean ± the standard error of the mean where n>2. In cases of small n numbers (3-5), the error of the mean is a predictive value to illustrate data variation only. In two-group data sets, paired or unpaired t-tests were used to analyse data. This was paired in situations where both data were collected from the same mouse. For data with multiple sets, two-way ANOVA with Bonferroni's post test was used. In some graphs with multiple data sets, clear trends were not significant in ANOVA tests. This was generally a result of low n numbers in one or more of the groups. Statistical significance was assumed where $p<0.05$.

CHAPTER THREE: EFFECTS OF ANGIOTENSIN II ON ARTERIAL BP AND CARDIOVASCULAR PARAMETERS OF HYPERTENSION IN WT AND CGRP KO MICE.

3.1 Introduction

It is well established that CGRP possesses vasodilator activity in the onset of vascular injury, however the effects on hemodynamics under normal physiological conditions was once less clear. As previously mentioned in the general introduction in chapter one, a number of studies have been carried out which utilize the genetic knockout models, however these have been shown to exhibit conflicting results in terms of the role of CGRP under basal conditions. Some groups reported no change in baseline BP in the mice after knockout of αCGRP, however others did report a significant increase in BP in the absence of αCGRP in comparison to their WT littermate controls (Table 1.2). It has however been identified that the difference in baseline BP was due to the different knockouts used. When blocking αCGRP alone, no BP increase is observed (Lu *et al.* 1999). However when calcitonin (CALC I) is also removed alongside αCGRP, a significant elevation in BP occurs, suggesting the increase to be calcitonin dependent (Gangula *et al.* 2000; Li *et al.* 2004; Bowers *et al.* 2005). Other studies have shown that injection of the CGRP receptor antagonists BIBN4096BS/$CGRP_{8-37}$ had no effect on systemic BP, which adds further evidence to the hypothesis that CGRP plays a minor role in BP regulation under basal conditions (Arulmani *et al.* 2004; Zeller *et al.* 2008; Olesen *et al.* 2004).

Ang II plays a key role in both the modulation of basal BP and the development of essential hypertension. It acts on various cell types to become a very powerful vasoconstrictor which in turn causes an elevation in BP, alongside increased blood volume, oxidative stress and the elevation of inflammatory mediators (Mehta and Griendling, 2007). The murine Ang II model of hypertension is now well established within our laboratory (Liang *et al*. 2009), taken originally from Byrne *et al.* in 1986. It involves the implantation of a small osmotic minipump containing Ang II subcutaneously into the mouse which osmotically infuses Ang II

for 14 days and subsequently induces hypertension. The onset of the hypertension in this model is quick and moderately severe, with some of the

characteristics mimicking human hypertension, but over a shorter space of time. These characteristics include vessel hypertrophy, cardiac remodelling and the induction of inflammation (Monassier *et al.* 2006).

CGRP release has been described as a key compensatory mechanism against elevating BP in both human and experimental models of hypertension, suggesting a protective role for the neuropeptide in cardiovascular disease (Bowers *et al.* 2005; Watson *et al.* 2011, Li and Wang, 2005, Deng and Li, 2005, Smillie and Brain, 2011). These studies have been extensively reviewed in chapter one, but to briefly summarise again, intravenous and systemic administration of CGRP into rodents causes hypotension in normotensive animals (Ando *et al.* 1990, Gardiner *et al.* 1991) and SHR (Gangula *et al.* 1999; Itabashi *et al.* 1988; Wimalawansa, 1996; Preibisz, 1993). Bolus administration of CGRP results in a rapid decrease in MAP in both WT and αCGRP KO mice, with an increased response in the αCGRP KO mouse, therefore resulting in a further decrease in MAP. In the calcitonin/αCGRP KO mouse, the hypertensive response to DOCA-salt induced hypertension was exacerbated compared to that observed in the WT mice. This increased hypertension was also accompanied by increased renal damage, therefore indicating a potentially protective role for CGRP in a hypertensive condition (Bowers *et al.* 2005).

3.2 Summary of the background

αCGRP KO mice have provided a valuable research tool to investigate the role of CGRP in BP regulation and disease. However the conclusions which have been drawn are dependent on the type of KO mouse created in the study. It is thought that primary deletion of αCGRP has no effect on basal BP. It is only when this deletion is combined with the deletion of calcitonin which then leads to an increase in basal BP. There is growing interest in CGRP, with an ever increasing number of papers being published every year describing the vasodilator nature of the neuropeptide, with further suggestions towards it being protective in the onset of cardiovascular disease. Due to the lack of mechanisms being published on how this peptide is protective, I have used WT and αCGRP KO mice to investigate the phenotype under basal conditions, before inducing hypertension via Angiotensin II

infusion to monitor any differences in the globally deleted αCGRP KO mouse compared to the WT.

3.3 Hypothesis

Under baseline conditions, CGRP will not play a role in the maintenance of BP. However, when infused with Ang II for 14 days, αCGRP KO mice will display an exacerbated hypertensive phenotype characterised by significantly elevated BP and end point cardiac hypertrophy.

3.4 Aims

- To investigate the role of αCGRP in the maintenance of BP under basal conditions.

- To investigate the role of αCGRP in the Ang II-induced model of hypertension.

    - Hypertension will be characterised by a significant difference in mean arterial BP, alongside evidence of cardiovascular parameters and organ weights.

3.5 Results

3.5.1 Investigating the basal characteristics of WT and αCGRP KO mice by measuring weight and BP using tail cuff plethysmography

Mice aged from 1 month were assessed for their increasing body weight over a period of 10 weeks whilst being fed a normal diet. Body weight was similar between WT and KO mice as shown in figure 3.1a, and did not vary significantly between genders, although males appear to be slightly heavier than females. Figure 3.1b shows that there is no significant difference in weight of both WT and αCGRP KO male mice at increasing weeks of age. Figure 3.1c shows that there is no significant difference in weight of both WT and αCGRP KO female mice at increasing weeks of age.

The basal BP of WT and αCGRP KO mice were established previously within our group using tail cuff plethysmography and therefore this was the primary choice of measurement for my study. The protocol and training regime for the BP recordings are described in detail in the materials and methods section. Figure 3.2 displays the baseline BP measurements for WT and αCGRP KO mice over a 14 day period. Figure 3.2a demonstrates that there is no significant difference in MAP between mixed gender WT and αCGRP KO mice. The mean of WT and αCGRP KO mice was 99.3± 0.41 mmHg. Figure 3.2b shows there is no significant difference in systolic pressure of both WT and αCGRP KO mixed gender mice, measured over 14 consecutive days. Mean systolic pressure in WT and αCGRP KO mice was 119.5 ± 0.53 mmHg. Figure 3.2c shows that there is no significant difference in diastolic pressure in both WT and αCGRP KO mixed gender mice, measured over 14 consecutive days. Mean diastolic pressure in WT and αCGRP KO mice was 79.3 ± 0.4 mmHg. This data is taken from daily readings and shows that there is no statistical difference in the BP of WT and αCGRP KO mice, suggesting that there is no difference in their phenotype under basal physiological conditions. The consistency of the readings over time also suggests that the mice became well trained quickly using the specific training regime and experimental conditions, therefore reproducible measurements were obtained.

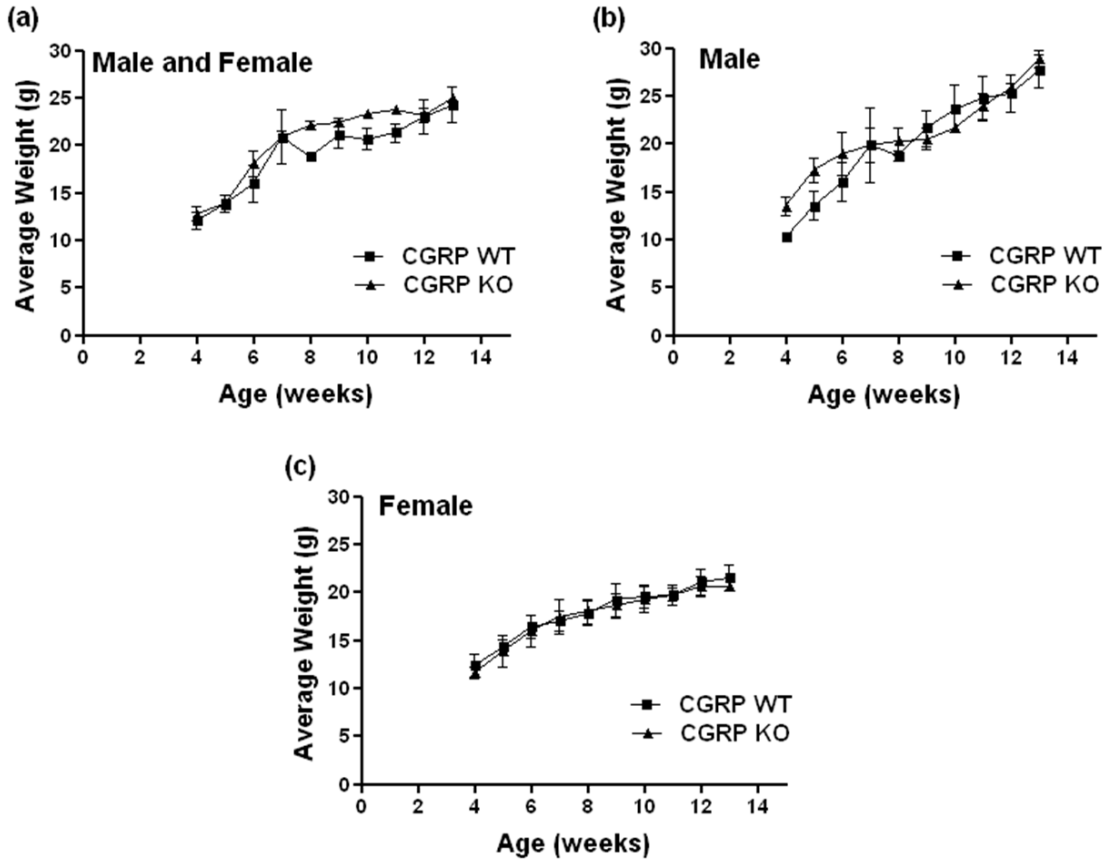


Figure 3. 1 Body weight of paired, littermate, WT and αCGRP KO mice measured over 14 weeks. Results show naive (a) Male and female WT and αCGRP KO mice (n=24), (b) male WT and αCGRP KO mice (n=12) and (c) female WT and αCGRP KO mice (n=12). Statistical evaluation of mean ± SEM by 2-way ANOVA + Bonferroni's test showed no significant difference.

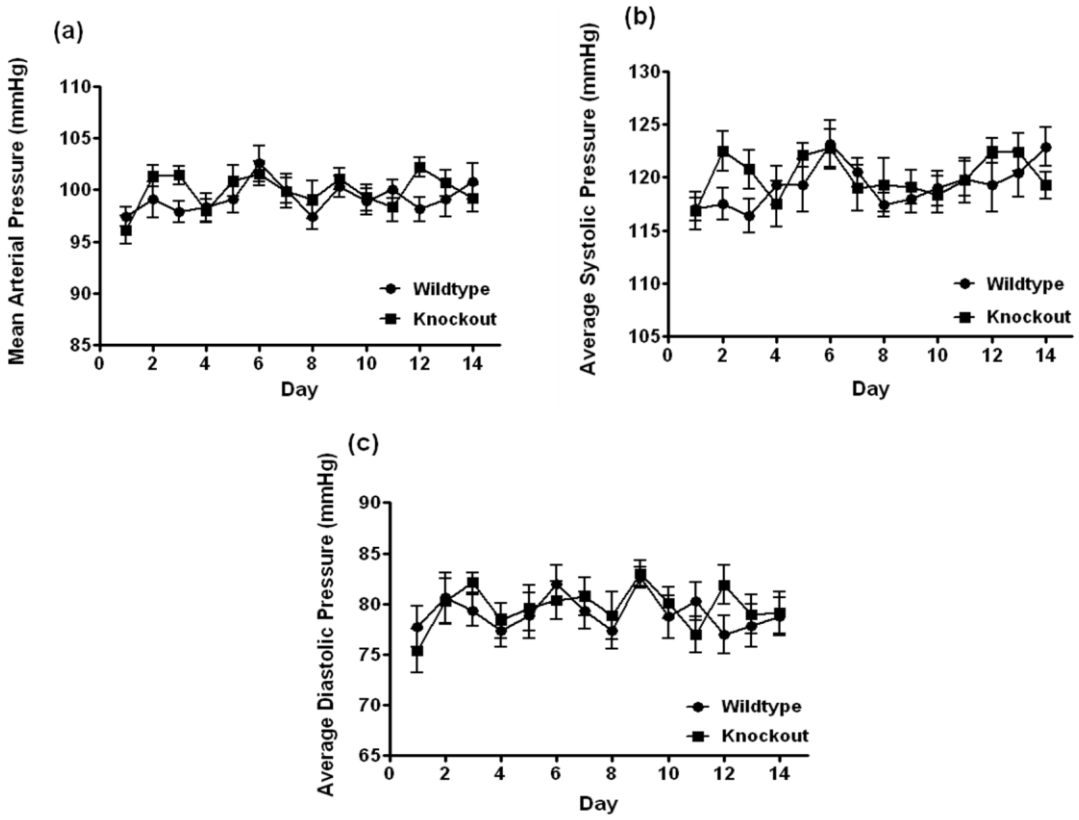


Figure 3. 2 Mean arterial pressure and average systolic and diastolic pressure (mmHg) of mixed gender WT and αCGRP KO mice over a 14 day time period measured by tail cuff plethysmography. Results show (a) mean arterial pressure (mmHg) in WT and αCGRP KO mice (n=12). (b) Average systolic pressure (mmHg) in WT and αCGRP KO mice (n=12) and (c) Average diastolic pressure (mmHg) in WT and αCGRP KO mice (n=12). Statistical evaluation of mean ± SEM by 2-way ANOVA + Bonferroni's test showed no significant difference.

3.5.2 Investigating the impact of Angiotensin II infusion for 14 days on BP and end organ weights of WT and αCGRP KO mice by measuring BP using tail cuff plethysmography

Figure 3.3 demonstrates arterial BP measurements, taken by tail cuff plethysmography, in WT and αCGRP KO mice over 14 days post Vehicle or Ang II infusion. Figure 3.3a shows that MAP (mmHg) is significantly elevated in the Ang II treated WT and αCGRP KO, with αCGRP KO mice being significantly higher than WT mice at days 7 and 9 ($p<0.01$ and $p<0.001$, 2 way ANOVA + Bonferroni's test respectively). Figure 3.3b demonstrates a significant increase in systolic pressure (mmHg) of both WT and αCGRP KO mixed gender mice infused with Ang II over 14 days. αCGRP KO mice had significantly higher systolic pressure compared to WT from days 7 to 14 ($p<0.01$ and $p<0.001$, 2 way ANOVA + Bonferroni's test respectively) Figure 3.3c suggests a trend towards a difference in the diastolic pressure in the Ang II treated WT and αCGRP KO mice, however after statistical analysis of 2 way ANOVA and Bonferroni's test, results showed no significant difference in diastolic pressure (mmHg) in both WT and αCGRP KO mixed gender mice, infused with either Vehicle or Ang II for 14 days.

The developmental markers of hypertension were assessed in mixed gender WT and αCGRP KO mice after 14 days infusion of either Vehicle or Ang II shown in Table 3.1. Significant differences were only observed between the 4 treatment groups in regards to heart to body weight ratio. The ratio was significant in WT mice infused with Ang II in comparison to their Vehicle treated counterparts. In addition, it was interesting to observe a significantly larger heart to body weight ratio in the WT Ang II treated mice in comparison to the Ang II treated KOs.

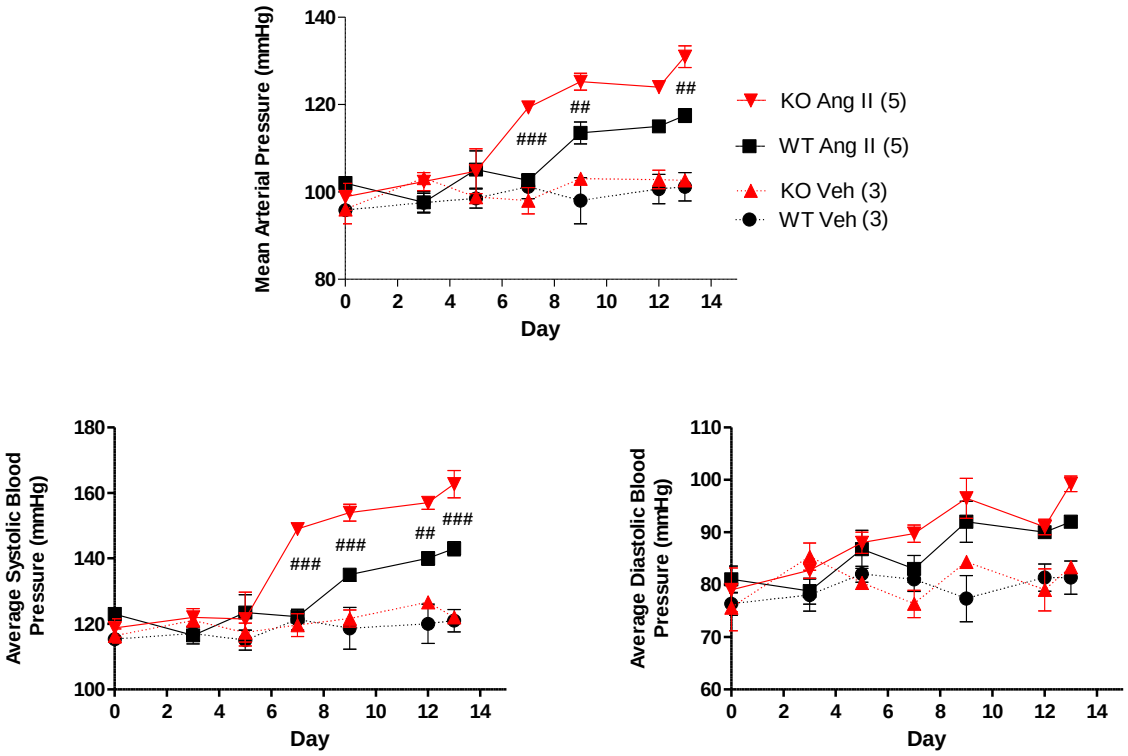


Figure 3.3 Mean arterial pressure (MAP) and average systolic and diastolic pressure (mmHg) of mixed gender WT and αCGRP KO mice treated with either Vehicle (saline) or Ang II infusion for 14 days, measured by tail cuff plethysmography. (a) Mean arterial pressure (mmHg) in WT and αCGRP KO mice, (n=3-5). (b) Average systolic pressure (mmHg) in WT and αCGRP KO mice, (n=3-5) and (c) average diastolic pressure (mmHg) in WT and αCGRP KO mice, (n=3-5). Statistical evaluation of mean ± SEM where #=p<0.05, ##=p<0.1 and ###=p<0.01 when compared to WT Ang II treated animals by two-way ANOVA + Bonferroni's test.

	WT Vehicle (n=9)	WT Ang II (n=10)	KO Veh (n=9)	KO Ang II (n=10)
Heart Weight (mg)	127.7±5.38	138.2±9.36	134.9±7.59	134.3±6.18
Left Ventricle Weight (mg)	18.88±1.51	18.55±1.79	22.43±1.48	17.36±1.01
Right Ventricle Weight (mg)	5.3±0.49	6.46±0.52	7.11±0.56	6.20±0.89
Heart:Body weight ratio	4.97±0.16	** # 5.85±0.23	4.66±0.10	5.21±0.10
LV:Heart	0.153±0.01	0.143±0.02	0.171±0.001	0.131±0.009
LV:Body	0.78±0.02	0.81±0.06	0.82±0.05	0.71±0.03
RV:Heart	0.042±0.004	0.047±0.002	0.053±0.043	0.047±0.007
RV:Body	0.19±0.02	0.27±0.01	0.27±0.02	0.25±0.03
Whole Kidney Weight (L and R, mg)	0.34±0.02	0.35±0.01	0.31±0.01	0.29±0.01

Table 3. 1 Effect of Angiotensin II Infusion for 14 days on endpoint markers of Hypertension in WT and αCGRP KO mice. Developmental markers of mixed gender, 8 week old WT and αCGRP KO mice after 14 days infusion of either Vehicle (saline) or Ang II (n=9-10). Statistical evaluation of mean ± SEM, by 2 way ANOVA and Bonferroni's test, where **=p<0.01 compared to vehicle treated WTs and #=p<0.05 compared to Ang II treated KOs.

3.6 Summary

- Age matched WT and αCGRP KO mice do not differ significantly in terms of body weight.
- Tail cuff plethysmography gave both consistent and reliable BP readings after undertaking a strict training regime. Using this technique, there was no statistical difference in BP in WT and αCGRP KO mice under basal conditions.
- Post 14 days Angiotensin infusion, BP is significantly elevated in both WT and αCGRP KO animals, however the hypertension was significantly exacerbated in the hypertensive αCGRP KO mice compared to hypertensive WT animals.
- Post 14 days Angiotensin infusion, heart to body weight ratio was significantly increased in WT mice compared to their vehicle controls, however this result was not shown in the αCGRP KO mice. No other significant findings in terms of end point cardiac or kidney weights were observed.

3.7 Discussion

This study has characterised the phenotype of WT and αCGRP KO mice under basal conditions in terms of body weight and baseline BP parameters. The phenotype was then further studied to identify any differences between the WT and αCGRP KO mice after 14 day Angiotensin II infusion to induce hypertension. This chapter has shown that WT and αCGRP KO littermates display a similar phenotype in terms of appearance, weight and BP.

3.7.1 WT and αCGRP KO mice show similar physical characteristics

WT and αCGRP KO mice bred in house, display similar physical characteristics and share similar weights at 4-14 weeks of age. Data is for mixed gender mice, in which there was no significant variance in weight dependent on gender, although males appear to be heavier than females at week 14. All mice displayed normal increases in weight in response to increasing age over 10 weeks. It has been noted that as mice age, all strains display gender based differences in body weight (growth charts available from JAX mice, jaxmice.org).

3.7.2 WT and αCGRP KO mice show similar baseline BP when measured by tail cuff plethysmography

The average BP of a mouse is similar to that of a human, being 120mmHg systolic and 80mmHg diastolic. Significant elevations or falls in these levels can be deemed as being dangerous and indicate hypertension and hypotension. As shown, tail cuff plethysmography was the choice of method to measure BP in this study. The method has been well established previously within our group and produces valid and reproducible data when following a strict experimental regime. It is therefore a well validated method to collect a series of BP measurements from conscious mice. Tail cuff plethysmography measures systolic pressure from the tail artery (Mahoney and Brody, 1978). This has been previously shown to give good comparisons to central BP; however there have been noted differences in the absolute pressure (Bunag and Riley, 1974). At basal levels (100-

120mmHg), the tail cuff is shown to give very similar readings to that of telemetry probes, however it has shown to differ at both lower and higher pressures (Mahoney and Brody, 1978) by a few mmHgs when looking at BP after induction of both hyper and hypotensive agents. Due to the telemetry procedure not being established within our laboratory at the point of conducting this study, it was more practical to conduct the study using tail cuff plethysmography. It should also be taken into consideration that the mice had already undergone one surgical procedure resulting in an implant (osmotic minipump), and this experimental approach avoided causing distress to the animal by conducting a second surgical procedure with the addition of a second implant and therefore two very large foreign objects in their bodies. Previous experience within the laboratory of these telemetry and Ang II mini-pumps is also suggestive of an increase in adverse events, compared with the negligible mortality rate and adverse events observed with tail cuff readings. There are advantages and disadvantages with this technique in comparison to telemetry, which is considered the gold standard which have previously been discussed in chapter two. However, as already mentioned, our strict training regime and taking care that all experimental conditions were tightly monitored to avoid variation ensured that our technique produced high quality and reproducible data.

Baseline BP was measured for a minimum 1-2 week period including training prior to minipump implantation to record baseline values. The results confirm that under baseline conditions, both WT and αCGRP KO mice display stable and reproducible measurements of a healthy 120/80mmHg, which we expected. This concurs with original data published by Lu *et al.* 1999, who created the C57BL/6 αCGRP KO mouse, and did not show any change in basal BP. Furthermore we have also shown in a separate project within our group that normal BP is observed in αCGRP KO mice until at least 15 months of age (King, Smillie and Brain, unpublished). On the other hand, this data does not support the findings by other groups who reported a higher basal MAP in the KO mouse compared to the WT as described in detail in table 1.2 from the general introduction (Li and Wang, 2005: Supowit *et al.* 2002; Gangula *et al.* 2000). Combining all of the results from these studies, it suggests that in our present findings, αCGRP does not have a role in the regulation of basal BP under normal physiological conditions.

A robust experimental plan was designed to ensure these measurements were reproducible, which involved carrying out the measurements blinded, having the results confirmed by additional measurements taken from another investigator and making sure that experimental conditions were kept constant throughout the study to minimise environmental variations.

3.7.3 Angiotensin II infusion for 14 days induces hypertension in both WT and αCGRP KO measured by tail cuff plethysmography

Here we have shown that after vehicle infusion, MAP does not differ between WT and αCGRP KO mice or from the baseline values. When mice were infused with Ang II, hypertension developed after 14 days in both WT and αCGRP KO mice compared to their matched vehicle controls. This is in keeping with studies such as Li and Wang who showed a significant increase in BP in the 7 week old male Wister rat after infusion of Ang II (100ng/kg/min) for ten days, (Li and Wang. 2005) and also previously in our group whereby 0.9mg/kg/day Ang II induced significant hypertension in C57BL/6 WT mice after 14 days (Liang *et al.* 2009). However, as hypothesised, the loss of αCGRP in the KOs resulted in exacerbated hypertension.

This also compliments data by Gangula *et al.* who also reported an exacerbated hypertensive response in the 129/57 αCGRP/calcitonin KO mice using the DOCA-salt induced model of hypertension (Gangula *et al.* 2000). Our result is novel in that it is the first reported study using the αCGRP KO mouse in the Ang II-induced experimental model of hypertension. However the role of CGRP in the BP increase during hypertension onset has been widely documented. In the Ang II study by Li and Wang, it was also shown that administration of the antagonist $CGRP_{8-37}$ significantly elevated the already elevated MAP in the hypertensive male Wistar rat. This was also accompanied by increased CGRP receptor expression in response to the Ang II induced hypertension, (Li and Wang. 2005). In addition to this, IV and systemic administration of CGRP has been shown to cause hypotension in SHRs (Gangula *et al.* 1999; Itabashi *et al.* 1988; Wimalawansa, 1996; Preibisz, 1993) and attenuates BP increases in DOCA-salt (Supowit *et al.* 1997), SN-salt (Supowit *et al.* 1998) and L-NAME (Gangula *et al.* 1997) induced models of hypertension as previously described in Chapter one.

Under normal physiological conditions, bolus administration of CGRP has been shown to result in a significant decrease in MAP in WT mice. However when administered to αCGRP/calcitonin KO mice, these animals showed increased sensitivity and therefore a further decrease in MAP is observed (Gangula *et al.* 2000). Bowers and co workers previously showed exacerbated hypertension in response to DOCA-salt treatment in αCGRP KO mice when compared to WT mice at both 14 and 21 days, however they were unable to identify key mechanisms as they could not elucidate whether the renal changes observed were dependent on an increase in BP, or whether it was αCGRP dependent (Bowers *et al.* 2005).

The data presented here along with the literature points to a key role for αCGRP in protecting against the onset of hypertension whereby αCGRP appears to be playing a compensatory vasodilator role to prevent the BP increase in Ang II induced hypertension.

3.7.4 Angiotensin II infusion for 14 days induces hypertension in both WT and αCGRP KO resulting in an increased heart to body weight ratio in WT but not αCGRP KO mice

In this study, heart to body weight ratio was significantly increased in WT Ang II treated mice in comparison to Ang II treated KOs. However, no other significant differences in organ weights were observed, which was surprising.

It is known that cellular inflammation plays a role in Ang II induced hypertension and vascular remodelling and fibrosis (Marchesi *et al.* 2008). We would therefore expect an increase in cardiac weight after 14 days. Calcitonin/αCGRP KO mice with DOC-salt induced hypertension displayed a 10% increased heart to body weight ratio in studies by Supowit *et al.* 2005 and Zhao *et al.* 2003, and increased renal damage alongside an increase in kidney mass (Bowers *et al.* 2005). These studies suggest that αCGRP has a protective action against hypertension induced heart and kidney damage, mediated directly by αCGRP or indirectly through subsequent neurohormonal or other physiological changes caused by the deletion of αCGRP. However other studies of hypertension have shown no significant increases in organ weight in hypertensive animal models, (Harrap *et al.*1993 and

Rohit *et al.* 2002). Possibilities for the lack of difference within the present study may be due to the number of days of infusion (14 days). Infusion periods have been shown to be as long as 28 days, (Jia *et al.* 2008 and Fitzgerald *et al.* 1997) and doses have been shown to be as high as 1.9mg/kg/day (Ishibashi *et al.* 2004). However, the absence of change in organ mass may not necessarily mean that there is a lack of pathophysiological or molecular changes occurring in the tissue.

3.8 Conclusion

In this chapter, I have shown that αCGRP KO mice do not display any phenotypical differences in terms of body weight and BP measured by tail cuff plethysmography under normal physiological conditions when compared to WT mice. When infused with angiotensin II for 14 days, BP significantly increases in both WT and αCGRP KO mice; however the hypertensive response is significantly exaggerated in the αCGRP KO mice. There is also a significant increase in heart to body weight ratio in hypertensive WT mice, however it was interesting to note that this result was not observed in the KOs, although they were more hypertensive. These results suggest that the presence of αCGRP helps to prevent the increasing elevated BP in hypertension, indicating a protective role for αCGRP in the onset of cardiovascular disease.

CHAPTER FOUR: THE EFFECTS OF ANGIOTENSIN II INFUSION FOR 14 DAYS ON CGRP AND ITS RECEPTOR COMPONENTS IN WT AND CGRP KO MICE

4.1 Introduction

CGRP exists in two forms, αCGRP and βCGRP, and although they are formed from two distinct genes on chromosome 11 in the human, they only differ by 3 amino acids, share 90% homology and possess similar biological properties (Steenbergh *et al.* 1986). As previously mentioned, αCGRP is distributed in the central and peripheral nervous system, whilst βCGRP is more commonly found in the enteric nerves (Mulderry *et al.* 1988). CGRP is a 37 amino acid neuropeptide with a structure composed of three defined sections as previously discussed in Chapter 1.

It is now well documented in the literature that CGRP acts on a unique and complex receptor in order to function at the cell surface, originally discovered by McLatchie *et al.* in 1998. The CGRP receptor belongs to the family B (secretin-like) G-protein coupled receptor in which CLR which must bind with a RAMP, in this case RAMP1. This association of RAMP1 with the CLR forms a functional CGRP receptor complex at the cell surface. RAMP2 binds with CLR to form an adrenomedullin receptor whilst RAMP3 has been known to form both adrenomedullin and intermedin receptors, and in some cases, a CGRP receptor. Without RAMP and CLR interaction, these key proteins are able to move to the cell surface independently, however they are non functional on their own (McLatchie *et al*. 1998).

The role and properties of CGRP and its receptor components in the cardiovascular system is now of great interest to many researchers. Known for its potent vasodilator activities, CGRP poses a protective role in ischaemic and other traumas. As already reviewed in the general introduction (Chapter 1), CGRP has been widely documented to be protective and of potential interest in terms of providing therapy to patients suffering from cardiovascular disorders. However the protective mechanisms are still not well understood, but thought to become important in vascular-related stress.

As discussed previously (Chapter One), the development of the αCGRP knockout mouse has caused some speculation with regards to its role in the maintenance of basal BP. Basal BP has been shown to be increased in αCGRP knockout models, however it has been identified that these models were also calcitonin absent, therefore this may be an indication as to why the basal BP was increased (See chapter 1). To date, the αCGRP knockout mouse developed by Salmon *et al.* and used throughout the duration of this PhD, do not display any basal BP differences when compared to their matched WT littermates, therefore we conclude that the αCGRP gene does not contribute to long-term BP regulation under basal conditions. However mouse models of hypertension using αCGRP knockouts have reported significant elevation in BP in hypertensive animals lacking in αCGRP in comparison to their WT littermates (Smillie and Brain, 2011), also including the results shown in this study as shown in the previous chapter.

In human studies, there is debate as to whether circulating plasma CGRP levels increase, decrease or remain unchanged in hypertension. Tang *et al.* (1989) and Portaluppi *et al.* (1992) showed decreased CGRP levels in plasma of patients with essential hypertension; however borderline essential hypertensive patients show no significant change in circulating plasma CGRP levels (Lemne *et al.* 1994). On the other hand, Masuda *et al.* (1992) showed increased plasma CGRP levels in hypertensive patients being restricted to a controlled sodium diet compared to normal patients. In addition to this, IV infusion of Ang-II (sequential doses of 8, 16, and 32ng/kg/min, each dose for 20 min) to 8 normotensive human males caused dose-dependent increases of circulating CGRP in addition to elevated BP (Portaluppi *et al.* 1993).

CGRP and its receptor have been shown to be expressed at elevated levels in experimental animal models of hypertension; however the role of CGRP depends on the experimental model chosen (See Chapter 1 for extensive review on this). Bolus injection of CGRP results in a decrease in BP, accompanied by increased plasma renin activity and plasma aldosterone in SHRs and Sprague-Dawley rats (Li and Wang, 2005; Gangula *et al.* 1999). RAMP1 over expressing mice have recently been shown to attenuate the increased BP and decreased baroreflex activity caused by Ang II infusion via an increased CGRP response, indicating a protective role for RAMP1 and CGRP in the cardiovascular system (Sabharwal *et al.* 2010).

In terms of the Ang II model of hypertension, in acute Ang II infusion, dose-dependent increases in BP and plasma CGRP concentrations occur, suggesting that Ang II and CGRP may not operate independently, but in fact interact with each other either directly or indirectly to regulate the cardiovascular system (Portaluppi *et al.* 1993). Supowit *et al.* (1995) had shown within their research group that CGRP mRNA levels were increased in dorsal root ganglia and the spinal cord in Mineralocorticoid-salt hypertension in male Sprague-Dawley rats. However when they repeated this study using a chronic Ang II infusion model over 14 days, CGRP mRNA expression did not significantly differ between the two groups of rats. Their studies therefore demonstrate that an increase in neuronal CGRP is not necessarily a result of increasing BP and they concluded that in this salt induced model of hypertension there are changes occurring in unidentified factors which therefore modulate the release of CGRP independently of BP elevation. (Supowit *et al.*1995).

A study by Li and Wang (2005) using male Wistar rats reported that Ang II infusion (100ng/kg/min for 10 days) was accompanied by an increase in CGRP receptor expression (CLR and RAMP1 in the mesenteric vessels) but not in CGRP synthesis and release (Li and Wang 2005). Bolus CGRP reduced mean arterial pressure in Ang II treated rats, whereas $CGRP_{8-37}$ increased MAP. However this study showed that CGRP receptor expression upregulation in the mesenteric vessels was indeed pressure dependent and to enhance the BP response to CGRP as Minoxidil (a K^{ATP}-channel activator) inhibited the BP increase in Ang II treated rats, and also abolished the increase in CLR and RAMP1 expression in mesenteric arteries.

4.2 Summary of the background

Both CGRP and the CGRP receptor have now been vastly studied in both human and rodent models of hypertension with a wide variety of results. Some models show elevation of CGRP in hypertension, whilst others do not. It is generally presumed from animal models that CGRP expression is raised with the onset of elevated BP; however it is still inconclusive as to whether this is due to a direct effect of the increased BP, or CGRP alone. Ang II infusion may perhaps alter the function of CGRP; however the role of CGRP and its receptor in the development of hypertension induced by Ang II remains unclear. This chapter of my PhD was

therefore designed to determine the role of CGRP and its receptor in Ang II induced hypertension. As Ang II infusion leads to an increase in BP, this elevated BP may confuse the results of CGRP and Ang II's interactions. On the other hand, elevated BP may in fact have direct effects on CGRP synthesis, release or CGRP receptor expression.

4.3 Hypothesis

Based on the literature, we hypothesised that circulating plasma CGRP levels would be elevated after 14 days Ang II infusion in comparison to vehicle treated animals, with mirroring results in terms of tissue mRNA expression. We also hypothesise that CLR and RAMP1 would be upregulated in hypertensive animals.

4.4 Aims

- To determine whether αCGRP mRNA and protein expression is increased, decreased or unchanged in WT hypertensive mice after Angiotensin II infusion for 14 days by RT-qPCR and ELISA.

- To investigate βCGRP mRNA expression in hypertensive WT and αCGRP KO mice after Angiotensin II infusion for 14 days by RT-qPCR.

- To determine circulating plasma CGRP levels in WT and αCGRP KO mice after vehicle or Ang II infusion for 14 days by ELISA.

- To investigate the CGRP receptor components (CLR and RAMPs) and determine any changes in mRNA expression after Angiotensin II infusion for 14 days by RT-qPCR and ELISA.

4.5 Results

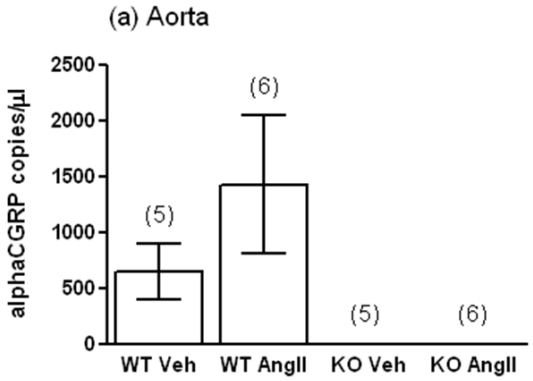
4.5.1 Investigating the effect of Angiotensin II infusion for 14 days on αCGRP and βCGRP mRNA expression in vascular tissues of WT and αCGRP KO mice.

After 14 days infusions with either Angiotensin II (1.1mg/kg/day) or vehicle (saline), mice were culled by cervical dislocation and organs and plasma harvested for analysis. For the purpose of this chapter of the study, aorta, heart, kidneys, MRV and DRG were carefully excised from the mouse and washed free of blood and immersed in RNAlater before being extracted for RNA and later, reverse transcribed to cDNA (see Chapter 2 for full methodology). RT-qPCR was then carried out to measure RNA expression of αCGRP, βCGRP and the CGRP receptor components in each tissue.

Figure 4.1 shows αCGRP mRNA expression in (a) the aorta and (b) heart, kidneys, MRV and DRG. All results are expressed as copies/µl of cDNA. As expected, no expression was found in CGRP KO mice, thus again confirming that the mice were indeed absent of αCGRP. αCGRP mRNA expression was upregulated in all tissues of hypertensive WT mice after 14 days Ang II treatment, particularly in MRV ($p<0.001$) and DRG ($p<0.05$) shown in figure 4.1b. However copy numbers in the MRV and DRG are relatively low, indicating low expression in this tissue, in comparison to the aorta which has high αCGRP expression when observing copies/µl alone (figure 4.1a).

Figure 4.2 illustrates βCGRP mRNA expression in the (a) aorta and (b) heart, kidneys, MRV and DRG. Again all results are expressed as copies/µl of cDNA. This time we did expect to observe expression in the αCGRP KO mice as the β isoform has not been knocked out in this model. In the aorta (figure 4.2a), βCGRP expression is reduced in the hypertensive animals (not statistically significant). However it is also worth noting that in the vehicle treated animals, although not statistically different, the αCGRP KOs had a higher basal expression of βCGRP when compared to WTs. In the heart βCGRP expression is upregulated in hypertensive animals, however it is significantly elevated in KOs after Ang II ($p<0.001$), with this elevation in expression being significantly higher than

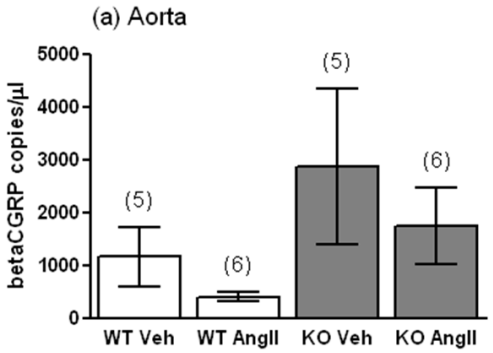
hypertensive WTs ($p<0.001$, Figure 4.2b). This trend is also mirrored in the DRG, although results are not significantly different (Figure 4.2b).



(b)

Organ	WT Veh	WT Ang II	KO Veh	KO Ang II
Heart	84.6±0.8	127.8±50.4	0.0±0.0	0.0±0.0
Kidney	469.9±385.5	1339±703.8	0.0±0.0	0.0±0.0
MRV	29.8±7.8	429.1±37.3 ***	0.0±0.0	0.0±0.0
DRG	24.9±0.7	48.3±7.0 *	0.0±0.0	0.0±0.0

Figure 4.1 Effect of Ang II (1.1mg/kg/day for 14 days) compared with vehicle (saline) on αCGRP mRNA expression in WT and αCGRP KO mice. αCGRP mRNA expression in the (a) aorta and (b) heart, kidney, mesenteric resistance vessels and dorsal root ganglia, measured by RT-qPCR, expressed as copies/µl and normalised to SDHA, HPRT-1 and PLA_2. *p<0.05 and *** p<0.001 compared to vehicle treated animals. N=3-6.



(b)

Organ	WT Veh	WT Ang II	KO Veh	KO Ang II
Heart	103.1±42.4	493.4±120.4	49.7±18.8	1900±234.8 *** ###
Kidney	$2.4x10^3±1.9x10^3$	$3.7x10^3±3.2x10^3$	$0.2x10^3±0.08x10^3$	$0.1x10^3±0.03x10^3$
MRV	$5.4x10^3±5.3x10^3$	$10.3x10^3±6.8x10^3$	$0.8x10^3±0.4x10^3$	$6.9x10^3±4.1x10^3$
DRG	$123.4x10^3±19.9x10^3$	$202.0x10^3±125.7x10^3$	$277.7x10^3±154.5x10^3$	$394.4x10^3±193.4x10^3$

Figure 4.2 Effect of Ang II (1.1mg/kg/day for 14 days) compared with vehicle (saline) on βCGRP mRNA expression in WT and αCGRP KO mice. βCGRP mRNA expression in the (a) aorta and (b) heart, kidney, mesenteric resistance vessels and dorsal root ganglia, measured by RT-qPCR, expressed as copies/µl and normalised to SDHA, HPRT-1 and PLA_2. *** $p<0.001$ compared to vehicle treated animals and ###$p<0.001$ compared to Ang II treated WTs. N=3-6.

4.5.2 Investigating the effect of Angiotensin II infusion for 14 days on circulating plasma CGRP levels in WT and αCGRP KO mice.

At day 14 of the study, and prior to cervical dislocation, mice were anaesthetised with 4% isoflurane (O_2 carrier gas, 4L/min flow rate) and a blood sample (1ml) was collected via cardiac puncture using a 25G needle from the left ventricle. This blood was then spun down for 10 minutes at 550xg, temperature controlled at 4ºC, and plasma was collected and snap frozen in liquid nitrogen. Plasma samples from each animal were extracted and purified before being assayed for CGRP detection by ELISA, an *in vitro* test which uses the quantitative sandwich enzyme immunoassay technique. The assay was obtained from Phoenix Pharmaceuticals, Germany and detected both mouse α and β CGRP isoforms. This was a rat/mouse CGRP EIA kit with 100% cross reactivity with rat and mouse and 15% cross reactivity with human and has previously been published by Murai *et al.* 2008.

Figure 4.3 illustrates the resulting plasma CGRP levels in WT and αCGRP KO mice after either vehicle or Ang II infusion for 14 days. After vehicle treatment, which we could also consider as a baseline value, circulating CGRP levels are similar in WT and αCGRP KO mice. However circulating CGRP levels increase in Ang II induced hypertension after 14 days in both WT and αCGRP KO's. Although statistical evaluation of the data does not show any significant difference, there is a trend occurring in hypertensive animals. However there is no difference between plasma CGRP levels in WT and αCGRP KO mice after 14 days Ang II infusion.

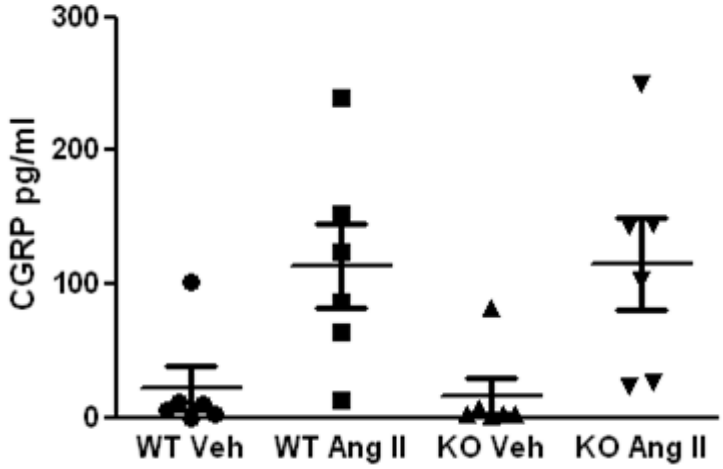


Figure 4.3 Effect of Ang II (1.1mg/kg/day for 14 days) compared with vehicle (saline) on circulating plasma total CGRP levels in WT and αCGRP KO mice. Circulating plasma CGRP levels, measured by ELISA and expressed as ng/ml. Statistical evaluation of mean ± SEM by ANOVA + Bonferroni's test showed no statistical difference. N=6.

4.5.3 Localisation of CGRP in the mouse aorta by Immunohistochemistry in both WT and αCGRP KO mice after either vehicle (saline) or Ang II infusion for 14 days.

After 14 days with vehicle or Ang II infusion, animals were killed by cervical dislocation and organs collected for post analysis. In order to observe aortic morphology the thoracic aorta was excised and carefully washed in saline before being fixed in paraformaldehyde and embedded in paraffin blocks. Sections of aortic rings were then cut on a microtome and stained for total CGRP by immunohistochemistry using the DAB-HRP reaction as described in Chapter 2. By looking at the morphology under the microscope we were able to identify clearly which cell types were positively stained for CGRP in comparison to the negative control (Figure 4.4a) as positive staining was brown. We could then observe any differences in staining between each treatment group; WT vehicle (b), WT Ang II (c), KO Veh (d) and KO Ang II (e). Densitometry was used to quantify total positive staining in the whole section (figure 4.4f), and also to separate by intensity of positive staining per cell type such as endothelial cell (g) smooth muscle cell (h) and surrounding collagen (i) as shown in Figure 4.4(g-i). Localisation of CGRP was found in all cell types, however after statistical evaluation there was no significant difference in CGRP expression in the aorta between WT and αCGRP KO mice, both after vehicle and Ang II infusion.

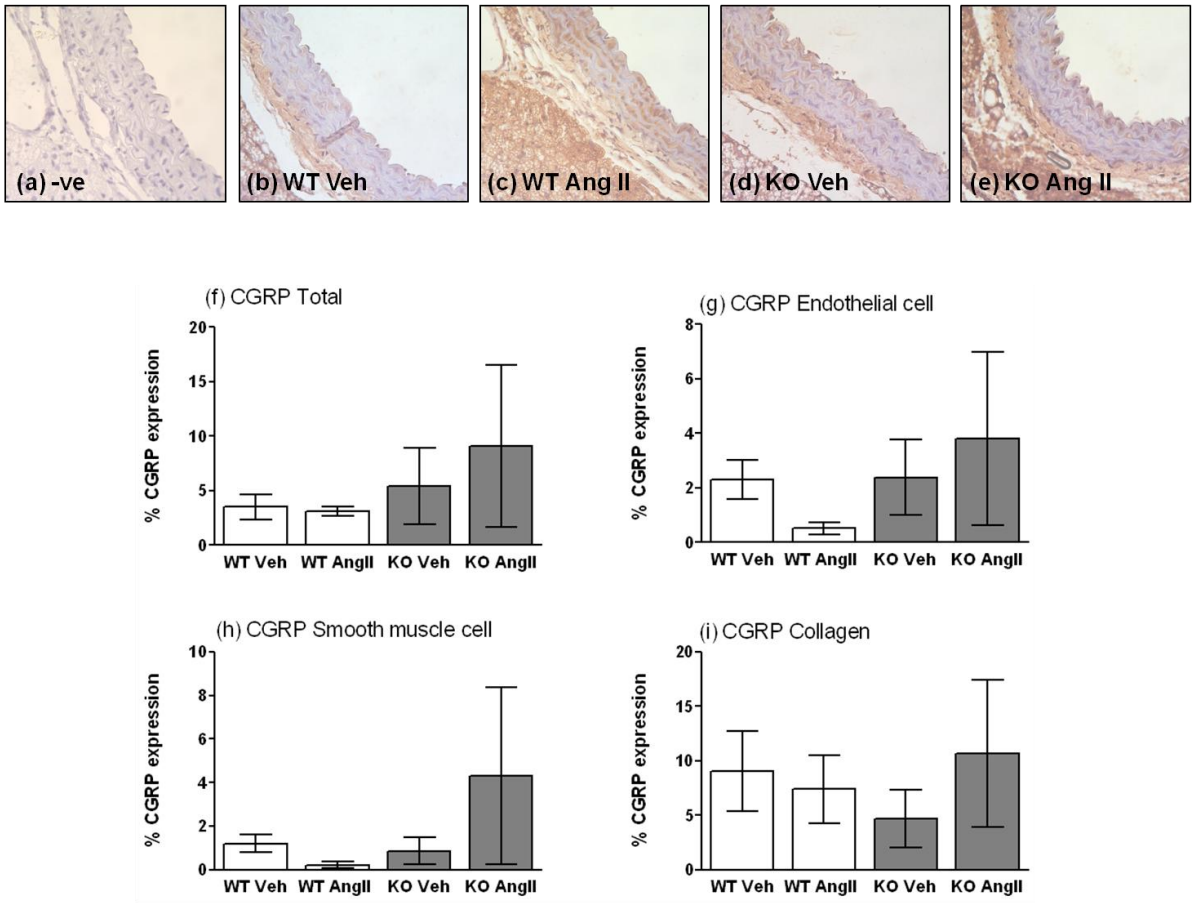


Figure 4.4 Localisation of immunoreactive CGRP in the aorta of WT and αCGRP KO mice and the effect of Ang II (1.1mg/kg/day for 14 days) compared with vehicle (saline) on CGRP expression in the aortas of WT and αCGRP KO mice. Immunohistochemical staining of CGRP in the thoracic aorta wall at 200x magnification. (a) negative control (b) WT vehicle (c) WT Ang (d) KO vehicle (e) KO Ang II and expressed as % positive CGRP expression of total thoracic section . Quantitative analysis of aortic CGRP expression in (f) the whole section (g) endothelial cells (h) smooth muscle layer and (i) surrounding collagen. Statistical evaluation of mean ± SEM by 2-way ANOVA + Bonferroni's test showed no statistical difference. N=4.

4.5.4 Investigating the effect of Angiotensin II infusion for 14 days on the CGRP receptor components mRNA expression in vascular tissues of WT and αCGRP KO mice.

As discussed in the introduction, the CGRP receptor is made up of the heterodimerization of CLR with RAMP1. As CGRP mRNA expression was increased in this model, and with previous reports of increased receptor expression in hypertensive animals, it seemed logical to look at the mRNA expression of the receptor components in this model in different tissues,

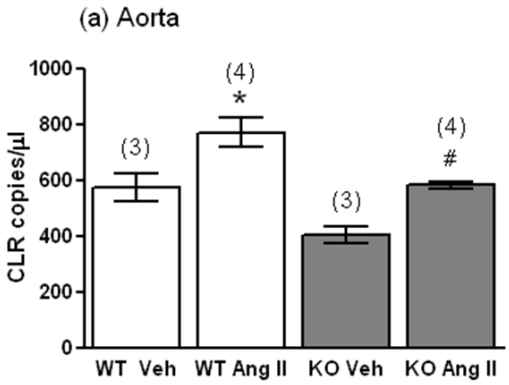
Figure 4.5 illustrates CLR mRNA expression in the aorta (a), heart, kidney, MRV and DRG (b) in WT and αCGRP KO mice treated with either saline or Ang II for 14 days. Again, all results are expressed as copies/µl of cDNA. In the aorta, CLR expression was increased in both hypertensive WT and αCGRP KO mice after Ang II infusion compared to their vehicle treated controls ($p<0.05$). This expression was significantly more elevated in WTs in comparison to the αCGRP KOs ($p<0.05$, Figure 4.5a). In the heart (Figure 4.5b), CLR expression was also increased in hypertensive animals, however again this increase in expression was significantly elevated in hypertensive WTs when compared to αCGRP KOs treated with Ang II ($p<0.001$). CLR mRNA expression was elevated slightly in both the kidney and MRV in the onset of hypertension, however no differences were observed in the DRG, although copy numbers were extremely low indicating low abundance of CLR expression in the DRG (Figure 4.5b).

Figure 4.6 shows RAMP1 mRNA expression in the aorta (a), heart, kidney, MRV and DRG (b). No differences in RAMP1 mRNA were observed in the aorta (figure 4.6a), MRV or DRG (figure 4.6b). Again, copy numbers were low, suggesting low abundance in these tissues. In the heart, RAMP1 expression was significantly upregulated in hypertensive WTs compared to their vehicle controls ($p<0.05$, figure 4.6b). No upregulation after Ang II infusion was observed in αCGRP KO mice, therefore RAMP1 expression was significantly elevated in Ang II infused WTs compared to Ang II infused αCGRP KOs ($p<0.001$). In the kidney, increased RAMP1 expression was only observed in KOs after Ang II infusion, being significantly elevated in comparison to hypertensive WTs ($p<0.05$, figure4.6b).

Figure 4.7 illustrates RAMP2 mRNA expression in the aorta (a), heart, kidney, MRV and DRG in WT and αCGRP KO mice treated with either saline or Ang II for

14 days, results expressed as copies/µl of cDNA. In all tissues, copy numbers were high, suggesting high abundance for RAMP2 expression in these tissues, however no significant differences were observed between WT and αCGRP KO mice after vehicle or Ang II treatment (Figure 4.7).

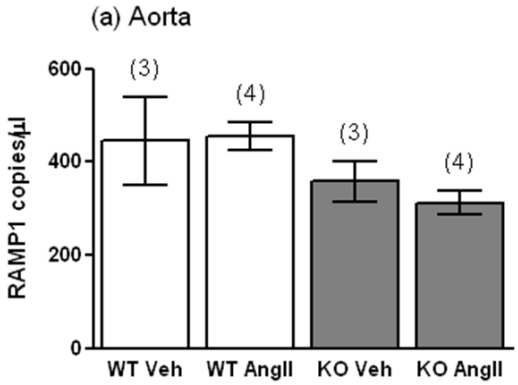
RAMP3 mRNA expression was also studied in the aorta, heart, kidney, MRV and DRG as shown in Figure 4.8. In this case, copies were low, suggesting low abundance of RAMP3 mRNA in these tissues. Again, no significant differences or trends were observed between WT and αCGRP KO mice after vehicle and Ang II infusion for 14 days (Figure 4.8).



(b)

Organ	WT Veh	WT Ang II	KO Veh	KO Ang II
Heart	$3.3x10^3±0.3x10^3$	$5.1x10^3±0.6x10^3$ *	$0.5x10^3±0.02x10^3$	$0.5x10^3±0.2x10^3$ ###
Kidney	572.3±12.5	664.7±128.1	496.7±68.4	570.1±53.3
MRV	27.4±26.4	89.3±46.9	25.4±16.0	48.8±16.9
DRG	19.5±8.8	10.1±3.5	9.2±2.7	10.0±0.9

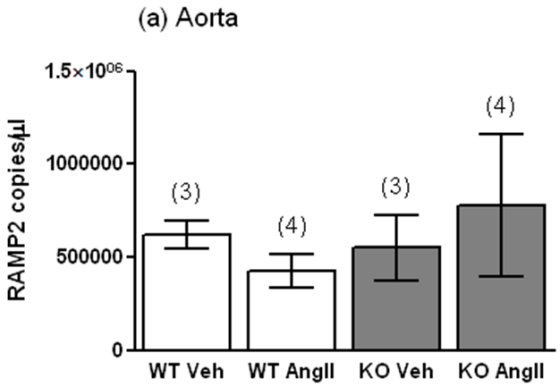
Figure 4.5 Effect of Ang II (1.1mg/kg/day for 14 days) compared with vehicle (saline) on CLR mRNA expression in WT and αCGRP KO mice. CLR mRNA expression in the (a) aorta and (b) heart, kidney, mesenteric resistance vessels and dorsal root ganglia, measured by RT-qPCR, expressed as copies/µl and normalised to SDHA, HPRT-1 and PLA_2. *p<0.05 compared to vehicle treated animals and #p<0.05 and ###p<0.001 compared to Ang II treated WTs. N=3-6.



(b)

Organ	WT Veh	WT Ang II	KO Veh	KO Ang II
Heart	$2.4x10^3 \pm 0.5x10^3$	$5.1x10^3 \pm 0.6x10^3$ *	$0.5x10^3 \pm 0.02x10^3$	$0.5x10^3 \pm x0.210^3$ ###
Kidney	$1.6x10^3 \pm 0.09x10^3$	$1.5x10^3 \pm 0.08x10^3$	$2.8x10^3 \pm 0.4x10^3$	$3.2x10^3 \pm 0.3x10^3$ #
MRV	31.73±1.9	35.0±2.6	33.8±3.0	30.7±0.6
DRG	33.74±4.0	30.35±1.7	36.3±1.2	32.8±3.3

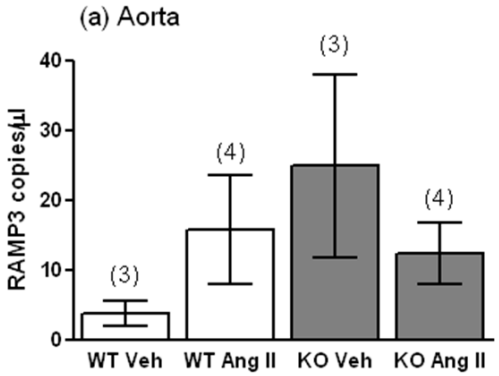
Figure 4.6 Effect of Ang II (1.1mg/kg/day for 14 days) compared with vehicle (saline) on RAMP1 mRNA expression in WT and αCGRP KO mice. RAMP1 mRNA expression in the (a) aorta and (b) heart, kidney, mesenteric resistance vessels and dorsal root ganglia, measured by RT-qPCR, expressed as copies/µl and normalised to SDHA, HPRT-1 and PLA_2. *p<0.05 compared to vehicle treated animals and #p<0.05 and ###p<0.001 compared to Ang II treated WTs. N=3-6.



(b)

Organ	WT Veh	WT Ang II	KO Veh	KO Ang II
Heart	$39.9x10^4±31.7x10^4$	$58.6x10^4±43.5x10^4$	$1.6x10^4±0.3x10^4$	$1.3x10^4±0.1x10^4$
Kidney	$29.5x10^4±27.8x10^4$	$3.8x10^4±3.6x10^4$	$5.2x10^4±2.4x10^4$	$2.4x10^4±2.0x10^4$
MRV	$26.4x10^4±24.5x10^4$	$92.3x10^4±41.5x10^4$	$5.4x10^4±2.5x10^4$	$45.0x10^4±25.0x10^4$
DRG	$12.3x10^4±1.4x10^4$	$11.3x10^4±2.5x10^4$	$13.5x10^4±13.4x10^4$	$14.1x10^4±3.5x10^4$

Figure 4.7 Effect of Ang II (1.1mg/kg/day for 14 days) compared with vehicle (saline) on RAMP2 mRNA expression in WT and αCGRP KO mice. RAMP2 mRNA expression in the (a) aorta and (b) heart, kidney, mesenteric resistance vessels and dorsal root ganglia, measured by RT-qPCR, expressed as copies/µl and normalised to SDHA, HPRT-1 and PLA_2. Mean ± SEM by ANOVA + Bonferroni's test showed no significant difference. N=3-6.



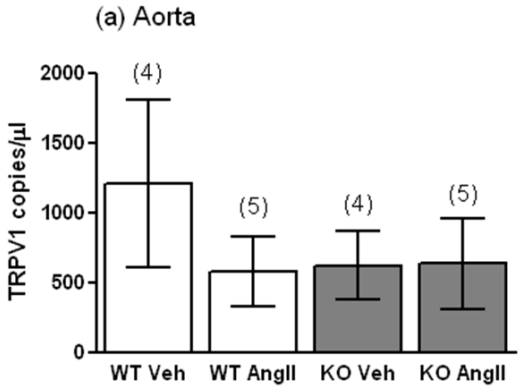
(b)

Organ	WT Veh	WT Ang II	KO Veh	KO Ang II
Heart	87.9±13.3	99.5±23.8	160.5±7.1	102.9±30.7
Kidney	19.5±10.0	19.9±8.0	12.8±6.1	19.1±2.7
MRV	340.6±212.4	410.7±206.9	167.7±19.1	415.4±172.9
DRG	439.7±140.0	376.2±98.5	329.2±142.4	402.1±83.5

Figure 4.8 Effect of Ang II (1.1mg/kg/day for 14 days) compared with vehicle (saline) on RAMP3 mRNA expression in WT and αCGRP KO mice. RAMP3mRNA expression in the (a) aorta and (b) heart, kidney, mesenteric resistance vessels and dorsal root ganglia, measured by RT-qPCR, expressed as copies/µl and normalised to SDHA, HPRT-1 and PLA_2. Mean ± SEM by ANOVA + Bonferroni's test showed no significant difference. N=3-6.

4.5.5 Investigating the effect of Angiotensin II infusion for 14 days on TRPV1 mRNA expression in vascular tissues of WT and αCGRP KO mice.

As previously discussed in the general introduction, CGRP has been shown to be released from sensory nerves following activation of TRPV1 in response to chemical stimuli. We were therefore interested to see if TRPV1 expression changed in response to Ang II infusion, and whether the absence of CGRP in the KOs would influence its mRNA expression. Results of this are shown in Figure 4.9 where TRPV1 mRNA expression was measured by RT-qPCR in the aorta (a), heart, kidney, MRV and DRG in WT and CGRP KO mice after either vehicle or Ang II infusion for 14 days. Copies/µl were observed in large numbers in MRV and DRG tissue, indicating high abundance for TRPV1 in these tissues compared to the heart and kidney, however the lack of CGRP in the KOs did not affect TRPV1 expression in vehicle treated animals. There was also no upregulation of TRPV1 expression after Ang II treatment in both WT and CGRP KO, suggesting that TRPV1 does not play a pivotal role in this particular model.



(b)

Organ	WT Veh	WT Ang II	KO Veh	KO Ang II
Heart	54.4±16.2	66.7±11.5	102.4±33.4	94.7±22.9
Kidney	46.4±4.2	47.7±17.5	35.5±0.9	27.7±7.3
MRV	$46.1x10^4$±$43.0x10^4$	$65.1x10^4$±$26.3x10^4$	$10.0x10^4$±$7.5x10^4$	$40.2x10^4$±$26.1x10^4$
DRG	$10.7x10^4$±$10.0x10^4$	$20.4x10^4$±$9.9x10^4$	$30.5x10^4$±$0.3x10^4$	$25.3x10^4$±$3.8x10^4$

Figure 4.9 Effect of Ang II (1.1mg/kg/day for 14 days) compared with vehicle (saline) on TRPV1 mRNA expression in WT and αCGRP KO mice. TRPV1 mRNA expression in the (a) aorta and (b) heart, kidney, mesenteric resistance vessels and dorsal root ganglia, measured by RT-qPCR, expressed as copies/µl and normalised to SDHA, HPRT-1 and PLA_2. Mean ± SEM by ANOVA + Bonferroni's test showed no significant difference. N=3-6.

4.6 Summary

- As expected, αCGRP mRNA expression was absent in αCGRP KO mice, confirming the deletion of this gene in the KO mouse

- αCGRP mRNA expression was upregulated in the aorta, heart, kidney, MRV and DRG in WT mice after Ang II infusion for 14 days.

- βCGRP mRNA expression is downregulated in the aorta after Ang II infusion, however it is upregulated in the heart and DRG

- Circulating plasma total CGRP show an increasing trend after Ang II infusion in both WT and αCGRP KO mice.

- Total CGRP protein expression in the aorta suggested localisation in the endothelium, VSMC and adventitia; however densitometry analysis showed no significant difference in CGRP expression in this tissue.

- CLR mRNA expression was significantly increased in the aortas of both WT and αCGRP KO after Ang II infusion. CLR mRNA expression was significantly increased in the hearts of WT but not αCGRP KO mice after Ang II infusion.

- RAMP1 mRNA expression was increased in the hearts of WT but not αCGRP KO animals after Ang II infusion. No trends were observed in other tissues, with low copy numbers suggesting low abundance of RAMP1.

- RAMP2 and RAMP3 mRNA expression did not change in the absence of CGRP or after Ang II infusion. Copy numbers suggested high abundance of RAMP2 in vascular tissues, but not RAMP3.

4.7 Discussion

4.7.1 αCGRP mRNA and circulating plasma total CGRP are increased after Ang II treatment and both α and β isoforms are located in the vasculature, particularly on endothelial and vascular smooth muscle cells of the aorta.

It has been shown previously that in humans, the majority of studies have concluded that circulating plasma CGRP levels are decreased in patients with essential hypertension compared to normotensive controls (Portaluppi *et al.* 1992; Tang *et al.* 1989; Wang *et al.* 2007). The data from these studies has therefore been suggested to be related to the role of CGRP in the pathophysiology of hypertension, possibly due to CGRP release being enhanced early in the process in order to counteract the increase in BP. They then suggest that after this increase, CGRP levels decrease. However, other studies report CGRP levels to be increased (Lind and Edvinsson, 2002), or even unchanged (Schifter *et al.* 1991). These differences in findings may be due to the varied choices in experimental analysis and sample preparation as shown previously in Table 1.1. With this inconclusive literature in mind, it seemed logical to look at CGRP levels in this model of hypertension, therefore RT-qPCR was used to measure α and β CGRP mRNA expression. Both isoforms were measured as a whole by ELISA for circulating plasma levels. In this study, I have shown that there is a trend towards an increase in circulating plasma CGRP in this model of hypertension after 14 days Ang II infusion. This also relates to the mRNA data in which we observed increased αCGRP expression in the aorta, heart, kidney, MRV and DRG of hypertensive WT mice (Figure 4.1). The absence of αCGRP mRNA expression in the αCGRP KO tissue again confirmed the genotype of the knockout mouse (Figure 4.1). There was also increased βCGRP in some vascular tissues (kidney and DRG, Figure 4.2b); however its expression was downregulated in the aorta (Figure 4.2a).

The ELISA assayed for both α and β CGRP in the plasma, hence the presence of total CGRP in KO animals (Figure 4.3). This was a rat/mouse CGRP EIA kit with 100% cross reactivity with rat and mouse and 15% cross reactivity with human. It is worth noting that this increase in CGRP expression, both at mRNA and plasma

protein level may indeed be time dependent. Perhaps this study has shown the CGRP levels increasing at 14 days, but these levels may perhaps change later on. Therefore a longer time point study would be beneficial to confirm this. This study was also achieved in the timeframe of this PhD and at 28 days, CGRP levels were further increased at both mRNA in the aorta, MRV and DRG and circulating plasma protein levels. These results will be further discussed in Chapter 7.

With regards to βCGRP, this isoform has not been studied in as great depth as αCGRP in the past. This appears to be due to people suggesting αCGRP is the most predominant isoform relevant to the cardiovascular system, being expressed in the whole central and peripheral nervous system, and that the βCGRP isoform was only found in the gut. (Poyner *et al.* 2002; Schutz *et al.* 2004; Smillie and Brain, 2011). It has also proved difficult to discriminate between the two peptides due to them being very similar and therefore successful separate α and β specific antibodies have not been available until very recently. In a study by Schutz *et al.* (2004) using the same C57BL/6 αCGRP KO mice as us, first generated by Salmon *et al.* (1999), they used RT-qPCR (SYBR green) and immunohistochemical techniques to compare α and β expression patterns in WT and αCGRP KO mice. In this study they found βCGRP expression in the DRG, however they report that in WTs, αCGRP mRNA was twice as abundant than βCGRP in this tissue (Schutz *et al.* 2004). This has also been previously shown in the DRG of the rat by Mulderry *et al.* in 1988 by chromatography and radioimmunoassay. Our results however indicate the opposite, with the βCGRP isoforms being the most abundant in the DRG of vehicle control WTs. However their study also reported that the βCGRP immunoreactivity was located in the small neurons. This data alongside our expression data therefore indicates that βCGRP is not just expressed in the gut, but also both in the vasculature and murine nervous system, which suggests a distinct role for the βCGRP isoforms in pain and neuromuscular systems. When observing the βCGRP mRNA expression in vascular tissue of Ang II treated animals in this study, we observed trends of downregulation in the aorta after Ang II infusion, however expression was slightly upregulated in the kidney and DRG. Also there were no differences between hypertensive WT and αCGRP KOs. This is novel data, and has not been studied previously. To refer back to the study by Schutz *et al.* they did not observe any

compensatory upregulation of βCGRP mRNA levels in the DRG of αCGRP KO mice. Again our results do not directly agree with this. Although not statistically significant, if we observe the data from Figure 4.2, we can see that βCGRP mRNA expression is upregulated in the aorta and DRG of vehicle treated αCGRP KO mice. This data may therefore that βCGRP is compensating for the lack of αCGRP in these animals. However in another study by Gangula *et al.* in 2000, it was shown that βCGRP was downregulated by more than 50% in the DRG of αCGRP KO mice compared to WT mice (Gangula *et al.* 2000). However this study was carried out in the combined CT/αCGRP KO mouse, which may influence results based on the original BP studies carried out on this strain. In conclusion to this data and the varying results found by different groups previously, different experimental methods of measuring CGRP expression may be the cause of different conclusions.

A study by Lu *et al.* (1999) failed to identify CGRP expression in the perivascular fibres of the aorta in the αCGRP KO mouse, which also suggested that βCGRP was not localised in the aorta, and therefore there was no compensation by the β isoforms for the lack of αCGRP in this tissue. This expression was also found to be minimal in the dorsal horn (Salmon *et al.* 1999) which therefore suggested that perhaps βCGRP was not detectable at all in the αCGRP KO mouse. This is definitely not the case in this model, which uses the same mouse as that by Salmon *et al.* as I have successfully detected βCGRP in circulating plasma, the aorta, heart, kidneys, DRG and MRV (see figure 4.2). With regards to the aorta, with the use of a polyclonal CGRP antibody raised in rabbit, provided by the Bunnett Group, USA, I have also successfully localised CGRP in the mouse aorta of both WT and αCGRP KO mice as shown in figure 4.4. As there is positive staining for CGRP in the αCGRP KO mouse, and due to the confirmation of the genotype of the αCGRP KO mouse carried out previously, we can assume that this positive expression shown is in fact βCGRP. When we observe the expression and quantify the data in both the WT and αCGRP KO mouse, CGRP is localised on both the endothelial and vascular smooth muscle cells. There is also positive staining seen around the adventitia and collagen. However when we quantify the positive staining, although there is no significant differences between the treatment groups, the findings are novel as we have successfully localised βCGRP in the aorta.

The results indicated in the previous chapter show that αCGRP KO mice exhibit enhanced hypertension. At present it is assumed this demonstrates a role for αCGRP in protecting against Ang II induced hypertension, independently of any role that βCGRP may have.

4.7.2 The CGRP receptor components- CLR and RAMP1 mRNA expression is only found to be increased in the heart of hypertensive WTs in this study, but not αCGRP KOs

It has previously been reported that the CGRP receptor is widely distributed in vascular beds including both small and large arteries, veins, capillaries, heart muscle cells and in the myocardium (Wilmalawansa, 1996; Hagner *et al.* 2002; Cottrell *et al.* 2005). This wide distribution of the CGRP receptor therefore may contribute to the enhancement of the depressor effect of CGRP and the upregulation of the CGRP receptor in cardiovascular diseases may be protective (Li and Wang, 2005). RAMP 1 is thought to be the predominant CGRP receptor when bound to CLR, and in a study by Sabharwal *et al.* (2010), RAMP1 overexpressing mice were shown to attenuate Ang-II induced hypertension, suggesting a protective role for both CGRP and its receptor components in the onset of hypertension (Sabharwal et al. 2010). CLR expression has also been reported to be upregulated in models of hypoxia (Nikitenko *et al.* 2003). Ang II infusion in Wistar rats has been shown to induce hypertension, in addition to increased CLR and RAMP1 protein expression in mesenteric arteries, however this increase in expression was shown to be pressure dependent as treatment with Minoxidil (an antihypertensive vasodilator) was shown to inhibit both the BP increase caused by Ang II and subsequently reduce receptor expression (Li and Wang, 2005). In this study, we did not observe increased receptor expression in the mesenteric resistance vessels, perhaps due to low expression being reported in our results for this particular tissue which may contribute to the lack of positive findings. However we did report an increase in CLR and RAMP1 mRNA expression in the aorta (Figure 4.5 and 4.6). CLR and RAMP1 mRNA expression was also significantly increased in hypertensive WTs but not αCGRP KOs, suggesting a possible protective role for αCGRP in myocardial protection.

RAMP 2 and the heterodimerization with CLR is known to form the functional receptor for Adrenomedullin, first identified by Kitamura *et al.* in 1993. Cardiovascular tissues are highly abundant with adrenomedullin and its receptor components, and this multifunctional regulatory peptide has vasodilatory and hypotensive properties (Beltoushi *et al.* 2004; Hinson *et al.* 2000). In previous reports, a study by Pan *et al.* (2010) using C57BL/6 adrenomedullin transgenic mice fed an atherogenic diet to induce hypertension showed that CLR and RAMP2 mRNA and protein expression was upregulated in the aortas of hypertensive mice, which correlated positively with an increase in aortic adrenomedullin mRNA expression and circulating plasma levels. These results suggested a protective role for adrenomedullin and the CLR and RAMP2 receptor components in hypertension (Pan *et al.* 2010). Similar findings were also reported by Kato *et al.* in 1997 who stimulated vascular smooth muscle cells with Ang II and then measured respective adrenomedullin and receptor levels. Protective responses of adrenomedullin, CLR and RAMP2 have again been reported in aortas of the spontaneously hypertensive rat, again suggesting positive roles for these peptides and receptors in cardiovascular protection (Pan *et al.* 2004). This group have also reported significant upregulation of RAMP3 mRNA and protein expression in both the spontaneously hypertensive rat and WT and AM transgenic C57BL/6 mice on atherogenic diets (Pan *et al.* 2004; Pan *et al.* 2010). With regards to this particular study, we did not identify upregulation of either RAMP2 (Figure 4.7) or RAMP3 (Figure 4.8) mRNA expression in this model in the onset of hypertension. However other groups utilising the Ang II model have reported increases in RAMP1 and RAMP3 expression in rat cardiomyocytes previously (Mishima *et al.* 2003). The lack of upregulation in RAMP3 expression (Figure 4.8) in our model suggests that RAMP1 is the more predominant RAMP for the CGRP receptor in our model of hypertension.

4.8 Conclusion

In this chapter I have shown that after vehicle infusion (baseline) there are no significant differences in circulating plasma CGRP levels or CGRP receptor mRNA expression in WT and αCGRP KO mice, suggesting that the receptor does not play a functional role under baseline conditions. However when infused for 14

days, circulating CGRP levels raise in both WT and αCGRP KO mice, alongside an increase in αCGRP mRNA expression in vascular tissues. Both CGRP isoforms are located in the mouse aorta as shown by immunohistochemical staining, however no differences were observed in WT and αCGRP KOs after Ang II infusion. The CLR and RAMP1 components of the CGRP receptor was shown to be elevated in the heart after Ang II infusion in WT animals but not KOs but in general, no other significant trends were observed. This increase in RAMP1 expression but not RAMP3 expression indicates RAMP1 being the predominant RAMP for the functional CGRP receptor in this model.

CHAPTER 5: THE EFFECTS OF ANGIOTENSIN II INFUSION FOR 14 DAYS ON VASCULAR HYPERTROPHY AND INFLAMMATION IN WT AND αCGRP KO MICE.

5.1 Introduction

The Ang II model has been well established previously within our laboratory to establish significant hypertension after 14 days (Liang *et al*. 2009). Not only does this model produce quick onset hypertension, but it has also been shown to mimic the human hypertension found clinically with regards to hypertrophy of the large blood vessels and heart, and the induction of associated inflammatory biomarkers (Monassier *et al.* 2006).

In a normal physiological state, the ROS producing enzymes play important signalling roles contributing to cellular activities such as growth, differentiation, migration and apoptosis which maintain homeostasis. However in the onset of a pathological condition such as hypertension, this redox homeostasis undergoes a shift towards an oxidising state, characterised by an increase in the production of ROS and depletion of the antioxidant defence mechanisms (Hitomi *et al.* 2007). This is called the induction of oxidative stress which will be discussed further in chapter 6.

The result of this oxidative stress also leads to the inflammation cascade and subsequent influx of inflammatory mediators which play a key role in the pathogenesis of vascular disease and the development of atherosclerosis. This inflammation is also present in hypertension. There is increasing evidence which suggests that the inflammatory response is induced by Ang II, through both BP dependent and independent mechanisms (Mervaala *et al.* 2000), where inhibition of the RAS with ACE inhibitors and AT1 receptor blockers and antagonists have depleted expression of the vascular inflammatory markers, making these effective in the treatment of hypertension and cardiovascular remodelling (Burnier *et al.* 2000; Cheng *et al.* 2003; Takai *et al.* 2003).

The inflammatory response is comprised of 3 stages. In the beginning (stage 1), in response to the stimulus such as Ang II there is an increase in vascular permeability. It is thought that Ang II increases this vascular permeability through

pressure dependent damage to the endothelium (Williams *et al.* 1995). The healthy endothelium produces NO derived from eNOS. When the RAS is activated, Ang II levels rise, which in turn is suggested to activate ET-1 and there is downregulation of eNOS, thus vasoconstriction occurs, leading to elevated BP and endothelial dysfunction. This then leads onto stage 2 of the inflammatory response which involves the infiltration of leukocytes from the circulation to the perivascular space. This is done through three stages: rolling, adhesion and transmigration as previously shown in Figure 1.4. from the general introduction in Chapter 1. During this phase, Ang II induces monocyte recruitment via expression of adhesion molecules of which VCAM-1 is key, and pro/anti-inflammatory cytokines onto the endothelium and progress into the VSMCs via activated NFκB. The cells then differentiate in the VSMC into macrophages in which production of pro-inflammatory cytokines and chemokines occur and a progressive inflammatory state develops within the vessel wall. The third and end stage of this process is tissue remodelling. This endpoint marks an overwhelmed inflammatory state which results in a change in vessel dimension and composition, often characterised by a marked difference in wall to lumen ratio, the onset of arterial stiffness and the formation of collagen deposits surrounding the outside of the vessel wall. To assess the three stages of vascular inflammation in the Ang II induced model of hypertension, selected markers of each stage were chosen and assessed by ELISA, immunohistochemistry or RT-qPCR. These markers are now discussed in turn.

5.2Stage 1- Vasoactive mediators (ET-1 and eNOS)

Endothelin-1 (ET-1) is a potent vasoconstrictor and regulator of regional blood flow under normal physiological conditions; however when it over-expressed in response to mediators such as Ang II, ET-1 acts on its receptors to induce contraction, proliferation and hypertrophy (Izzo *et al.* 2008). This contractile response contributes to the onset of hypertension, and it is thought that it also participates in the onset of vascular injury through increased oxidative stress and stimulation of pro-inflammatory markers such as interleukins, VCAM-1, MCP-1 and ICAM-1 (Duerrschmidt *et al.* 2000; Luft *et al.* 1999; Cheng *et al.* 2005). The connection between Ang II and ET-1 is contradictory, and perhaps model dependent, with studies reporting increased ET-1 expression in Ang II stimulated

VSMCs of rats, however this is not so in hypertensive salt-sensitive mREN2 rats (Rajagopalan *et al.* 1997; Moreau *et al.* 1997; Rossi *et al.* 2000). In these studies it was also shown that ET-1 receptor antagonists reduced BP and inhibited vascular inflammation in the Ang II treated rats but not the mREN2 rats. However in other models of hypertension, ET-1 has been shown to play a positive role in the development of hypertension in rats induced by DOCA-salt (Larivière *et al.* 1993; Schiffrin *et al.* 1995), L-NAME (Verhagen *et al.* 1998) and the 2K1C models (Sventek *et al.* 1996). Studies by Meens *et al.* (2009) have shown CGRP to relax contractile responses to ET-1 suggesting a strong connection between these two mediators and the onset of hypertension which will be discussed further later on in this chapter, therefore it seemed important to look at ET-1 expression in this model.

Endothelial nitric oxide synthase (eNOS) generates NO via L-arginine metabolism within the blood vessels which regulates vascular tone (BP control) and pressure-natriuresis under normal physiological conditions. This NO inhibits contraction and proliferation of VSMCs through vasodilatation, which can be increased in response to shear stress and increased vasoconstrictor agents such as ET-1. It mediates its vasodilatory actions through activation of guanylate cyclase. This induces relaxation of the smooth muscle either by increased cGMP which inhibits calcium entry and thus decreases intracellular calcium concentrations, or by activation of potassium channels which then leads to hyperpolarisation and relaxation (Izzo *et al.* 2008). Abnormalities in endothelial production of NO result in endothelial dysfunction and the progression of hypertension, vascular remodelling and atherosclerosis (Moncada *et al.* 1993). A study by Zhao *et al.* showed that gene therapy with human eNOS decreased fructose-induced hypertension in rats via increased NO and intracellular cGMP production (Zhao *et al.* 2008). eNOS mutant mice have been shown to have a hypertensive phenotype under baseline conditions (Huang *et al.* 1995) and inhibition of eNOS in Apo-E-deficient mice using double knockout mice results in significant aortic atheroma formation and elevated BP (Knowles *et al.* 2000) therefore indicating a pivotal role for eNOS in the maintenance of basal vasodilatation and BP.

5.3 Stage 2- Adhesion molecules and chemokines (MCP-1, VCAM-1, ICAM-1)

Leukocyte migration in response to Ang II upregulation is mediated by adhesion molecules in the progression towards vascular inflammation, remodelling and atherosclerosis (Sanz-Rosa *et al.* 2005). Increased expression of MCP-1, VCAM-1 and ICAM-1 have been observed in the vessel walls in the SHR, Ang II infused rats and in both rodent and humans with atherosclerosis (De Ciuceis *et al.* 2005; Ando *et al.* 2004; Yla-Herttuala *et al.* 1991). In addition to this, serum levels of these adhesion molecules are also raised in patients with systemic vascular inflammation (Rohde *et al.* 1998; Aukrust *et al.* 2001).

MCP-1 is a chemokine expressed by vascular cells including the endothelium, VSMC and cardiomyocytes. It plays an important role in the recruitment of monocytes into the tissue when upregulated by an inflammatory stimulus including Ang II and has been associated with fibrosis and tissue remodelling, therefore making it a key biomarker in both tissue and circulating plasma in the onset of inflammatory diseases. In a model of Ang II induced hypertension, Behr *et al.* (2004) showed that an AT1 antagonist (Eprosartan) reduced MCP-1 expression in SHRs, which in turn reduced inflammation due to inhibited infiltration of macrophages (Behr *et al.* 2004). Inhibition of MCP-1 by transfecting the deletion mutant of human MCP-1 gene limited Ang II induced progression of atherosclerotic lesions and suppressed the induction of pro-inflammatory genes in Ang II infused rats (Ni *et al.* 2004). Myocarditis and vascular and cardiac remodelling has predominantly been found in MCP-1 overexpressing mice, therefore indicating an important role for MCP-1 and inflammatory cell migration in initiating a pro-inflammatory response within the cardiovascular system (Kolattukudy *et al.* 1998).

Intracellular Adhesion Molecule 1 (ICAM-1) is a surface glycoprotein expressed on the endothelial cells and cells of the immune system. Under normal physiological conditions this adhesion molecule is present continuously in low concentrations in the membranes of leukocytes and endothelial cells. However, in response to various stimuli, ICAM-1 expression on the endothelium significantly elevates (e.g. in response to IL-1 and TNF-α activation). During the inflammatory

process leukocytes are able to bind to the endothelium via activated ICAM-1 and then transmigrate into the tissue (Yang *et al.* 2005).

Vascular cell adhesion molecule protein 1 (VCAM-1) also functions as a cell adhesion molecule expressed on the endothelium alongside ICAM-1 and the two distinct proteins share similar properties. VCAM-1 is responsible for the adhesion of lymphocytes, monocytes, eosinophils and basophils to the vascular endothelium. Again like ICAM-1, expression of this adhesion molecule is present under normal physiological conditions, and this is elevated in response to inflammatory mediators such as TNF-α and IL-1 (Yonekawa *et al.* 2005).

Both VCAM-1 and ICAM-1 are detected in plasma under baseline conditions; however these levels have shown to be elevated in the onset of inflammatory cascades such as heart transplant rejection (Ballantyne *et al.* 1994) atherosclerotic formation in the aorta (Nakai *et al.* 1996) and also in human essential hypertension (De Souza *et al.* 1997) and diabetes induced hypertension (Gasic *et al.* 1999). Cultured VSMCs and endothelial cells treated with Ang II showed enhanced VCAM-1 and ICAM-1 expression via NF-κB activation by Ang II induced oxidative stress (Pueyo *et al.* 2000; Pastore *et al.* 1999). Increased ICAM-1 and VCAM-1 expression have also been observed in the mesenteric vessels, the heart and the aorta of rats treated with Ang II compared to controls (Alvarez *et al.* 2004; Diep *et al.* 2002; Kiarash *et al.* 2001; Tummala *et al.* 1999), making these ideal markers to study in our model of hypertension.

5.4 Stage 2- Cytokines (Interleukins and TNF-α)

The cytokines are small intracellular cell-signalling protein molecules produced from monocytes and macrophages in response to an inflammatory stimulus such as oxidative stress. They respond by enhancing cellular immune responses, and are readily present in circulating plasma in disease, thus making them important markers of inflammation to assess.

IL-1 is a general name for two different cytokines, named IL-1α and IL-1β, however due to their similar pro-inflammatory roles they are generally referred to as IL-1. They are primarily produced from monocytes and macrophages, however studies have also reported IL-1 to be expressed by fibroblasts (Fini *et al.* 1994),

hepatocytes (Tsukui *et al*. 1994) and brown fat adipocytes (Burysek and Houstek, 1996). Within the immune system, the production of IL-1 is known to result in the onset of inflammation; however IL-1 has also been associated with bone formation and remodelling (Kusano *et al.* 1998; Dodds *et al.* 1994). Plasma levels of IL-1 are elevated in patients with coronary artery disease (Waehre *et al*. 2004). In hypertension, IL-1 has been shown to enhance the hypertensive effects of Ang II by upregulating the AT_1 receptor expression on VSMC, with the possibility that this may be via interference with NO regulated vascular tone (Sasamura *et al.* 1997).

IL-6 is primarily expressed in macrophages, however it has also been known to be produced in fibroblasts, VSMC and endothelial cells. It has been linked to hypertension, being a positive marker for end organ damage and severity, however it is an interleukin which is known to be both pro-inflammatory and anti-inflammatory. It is thought to play an anti-inflammatory role through the inhibition of TNF-α, and activation of IL-10 and PAI-1. The role of IL-6 as an inflammatory marker of inflammation has been well documented in models of cardiovascular dysfunction. Increased IL-6 expression is followed closely by increased macrophage activation and adhesion molecule over expression in the vascular wall (Ikeda *et al.* 1993). In normotensive rats, IL-6 infusion caused fibrosis and hypertrophy in the heart and surrounding vessels, independently of BP (Melendez *et al.* 2010). Increased levels of IL-6 have also been reported in the SHR (Haugen *et al.* 2008) and following Ang II infusion in WT mice (Manhiani *et al.* 2007). In addition to this, hypertension has been shown to be attenuated in the IL-6 KO mouse, however the mechanisms are unclear, but thought to be through an IL-6 dependent mechanism which is independent of renal injury (Manhiani *et al.* 2007). These studies suggest a positive role for IL-6 in the onset of hypertrophy and vascular/cardiac remodelling which is reversed by inhibition of this cytokine, as shown through the use of the IL-6 KO mouse.

IL-8 is another chemokine produced by macrophages in response to stress and its role is to attract neutrophils at the site of inflammation. The mouse homologue of IL-8 is KC which will be investigated in this study. This chemokine was chosen based on evidence supporting a role for IL-8 in neutrophil activation in the aetiology of atherosclerosis (Hastings *et al.* 2009) via Ang II Nabah *et al.* 2004)

and in the SHR (Kim *et al.* 2008). Ang II infusion has also been shown to increase IL-8 expression in human VSMCs (Ito *et al.* 2002).

IL-10 is one of the anti-inflammatory cytokines produced by macrophages, monocytes and lymphocytes. This cytokine has been shown to reduce inflammation, endothelial dysfunction and BP in DOCA-salt induced hypertensive pregnant rats, perhaps through downregulation of circulation ET-1 (Tinsley *et al.* 2010). IL-10 has also been shown to inhibit Ang II induced vascular dysfunction in Ang II treated carotid rings from WT and IL-10 KO mice (Didion *et al.* 2009).

IL-12 is a pro-inflammatory interleukin produced by dendritic cells and macrophages in response to stimulation by an antigen. This cytokine is thought to play a key role in the development of atherosclerotic plaque formation (Uyemura *et al.* 1996; Hauer *et al.* 2005). Again, in DOCA-salt induced hypertensive rats, IL-12 expression was upregulated alongside IL-10 (Tinsley et al. 2009). IL-12 has also been shown to be upregulated in Ang II-accelerated atherosclerosis in WT mice (Bruemmer *et al.* 2003). This upregulation has also been shown in the circulating plasma from patients with Type 2 diabetes (Wegner *et al.* 2008).

TNF-α is one of the most widely documented cytokines, with its role in tumor necrosis induction and inflammatory diseases being well characterised. It is produced from macrophages, VSMC, endothelial cells and fibroblasts (Thalmann and Meier, 2007). When produced, TNFα and IL-1 induce activation and recruitment of adhesion molecules including VCAM-1 and ICAM-1, both which promote monocyte adhesion to the endothelial cells via MCP-1 activation, triggering an inflammatory cascade at the site of injury (Savoia and Schiffrin, 207). TNFα promotes endothelial dysfunction through the inhibition of the vasodilatory actions of NO in the vasculature via activation of superoxide production from endogenous xanthine oxidase to promote ROS-induced apoptosis (Zhang, 2008). In models of hypertension, overexpression of TNF-α is associated with decreased vascular endothelial growth factor (VEGF) and severe pulmonary hypertension compared to WT mice, although precise mechanisms still remain to be elucidated but may be due to decreased VEGF gene expression (Fujita *et al.* 2002). Also in a model of arthritis induced hypertension, patients treated with TNF-α antibodies over 3 months showed a reduction in BP alongside a reduction in systemic inflammation. This is thought to be mediated by the improvement in microvascular

endothelial-dependent functioning (Sandoo *et al.* 2011). These studies characterise a pro-inflammatory role for TNF-α in the onset of hypertension and inflammation.

5.5 Stage 3- Collagen deposition

The last stage of vascular inflammation is remodelling and end point damage to the vessel wall which is reversible in early remodelling, however if left untreated, over time this remodelling progresses and becomes irreversible without surgical intervention. In hypertension, this damage is characterised by increased vessel wall to lumen ratio and arterial stiffness. There is also the formation of collagen surrounding the vessel and in progressive cases, plaque and foam cell formation within the vessel in atherosclerotic formation (Izzo *et al.* 2008; Robert *et al.* 1994).

Collagens are a group of proteins produced mainly from fibroblasts and found naturally in the skin, connective and fibrous tissue of animals and humans. It is the most abundant protein in mammals, making up nearly 30% of the body's total protein content (Di Lullo *et al.* 2002). However under inflammatory conditions, collagen can be produced from fibroblasts in excess and cause detrimental effects to the vessel walls. There are 28 types of collagen, however the five most common are collagen I which is found in the skin, collagen II which is predominantly found in cartilage, collagen III which is the collagen of granulation found in arterial walls, collagen IV in the cell basement membrane and collagen V which is present in hair (Hulmes, 1992). Collagen III will be assessed in this study due to it previously being shown to be expressed in the aortic vessel and hearts in models of hypertension including aldosterone-salt induced hypertension in rats (Robert *et al.* 1994); coarctation of the rat abdominal aorta (Burgess et al. 1996) and in human essential hypertension (Pardo-Mindan *et al*. 1993). Ang II has also been shown to induce collagen synthesis in cultured fibroblasts (Guarda *et al.* 1993).

5.6 Summary of the background

In this introduction I have described how during the onset of hypertension, impaired endothelial NO production and ET-1 mediated vasoconstriction causes

elevation in BP. This is then followed closely by systemic inflammation, characterised by the expression of cytokines and adhesion molecules which cause an exacerbated inflammatory response which, if left unresponded, can lead to end point vessel remodelling and atherosclerosis. The levels of some of these markers of inflammation will be investigated in this chapter as a measure of severity in this model. All markers which will be assessed have previously been reported to be upregulated in the onset of hypertension, particularly in models induced by Ang II infusion.

5.7 Hypothesis

In the previous chapter I have shown that Ang II induces significant hypertension in WT mice after 14 days. However this hypertensive response was exaggerated in the absence of αCGRP. We hypothesise that in hypertensive WT mice, the hypertension has induced the elevation of inflammatory markers and the onset of vascular hypertrophy. However we suspect that this inflammation and vascular remodelling may perhaps be exacerbated in αCGRP KO mice.

5.8 Aims

- To investigate vasoactive mediators including endothelin-1 (ET-1) and endothelial nitric oxide in the hypertensive WT and αCGRP KO mouse after Angiotensin II infusion for 14 days by real time pcr (RT-qPCR). Circulating ET-1 levels will also be assessed by ELISA.

- To assess markers of vascular adhesion molecules and chemokines (VCAM-1, ICAM-1 and MCP-1) in both the WT and αCGRP KO mouse and identify a role for αCGRP in the onset of Ang II induced inflammation.

- To investigate the role of αCGRP in the onset of vascular inflammation in the Ang II-induced model of hypertension by analysing the plasma

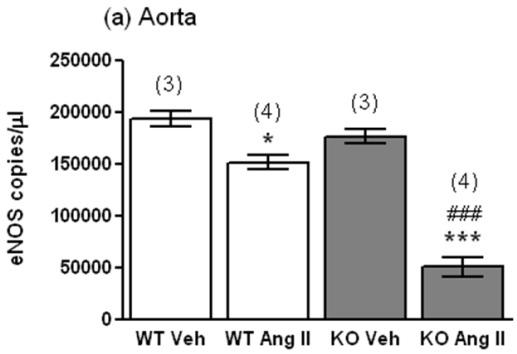
inflammatory cytokine profile (IL1, 6, 8, 10, 12 and TNF-α) of the WT and αCGRP KO mouse.

- To investigate the role of CGRP in the onset of vascular hypertrophy in the aorta after 14 days Ang II infusion using histological techniques to observe the morphology of the aorta in WT and CGRP KO mice.

5.9 Results

5.9.1 The effect of vehicle or Angiotensin II infusion for 14 days on endothelial nitric oxide synthase (eNOS) mRNA expression in vascular tissues of WT and αCGRP KO mice, measured by RT-qPCR.

eNOS mRNA expression as shown in Figure 5.1 was determined by RT-qPCR in the aorta (figure 5.1a), heart, kidney, MRV and DRG (figure 5.1b) in WT and αCGRP KO mice after 14 days of vehicle or Ang II infusion. In the aorta, eNOS expression was significantly downregulated in both WT ($p<0.05$) and αCGRP KO ($p<0.001$) mice after Ang II infusion when compared to their matched vehicle treated controls. However in the hypertensive αCGRP KO mice this expression was significantly lower again when compared to Ang II treated WTs ($p<0.001$), perhaps suggesting an increased response to the onset of endothelial dysfunction in these animals due to their significantly elevated hypertensive response to Ang II (Figure 5.1a). With regards to the other vascular tissues measured, eNOS expression was again downregulated in the heart, kidney and MRV after Ang II infusion, however unlike the aorta, this was only significantly reduced in αCGRP KO animals ($p<0.01$) and no statistical difference was observed between the two hypertensive groups of mice (Figure 5.1b). No difference in gene expression was observed with regards to the DRG (Figure 5.1b).



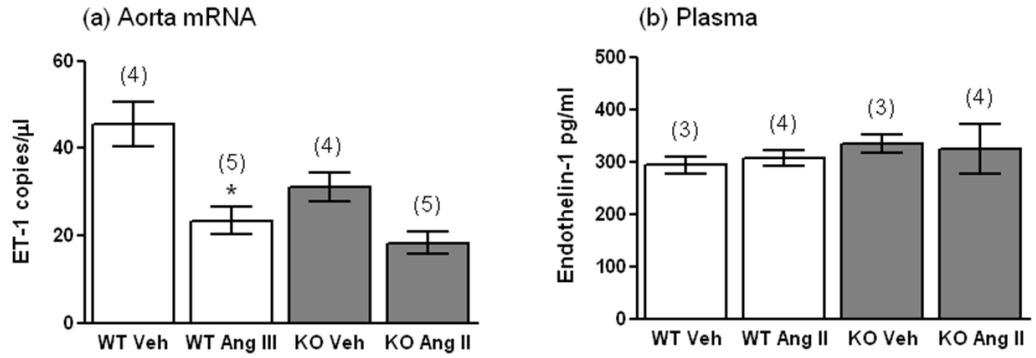
(b)

Organ	WT Veh	WT Ang II	KO Veh	KO Ang II
Heart	$198.0x10^4 \pm 33.9x10^4$	$121.5x10^4 \pm 10.5x10^4$	$225.8x10^4 \pm 25.0x10^4$	$87.4x10^4 \pm 1.9x10^4$ **
Kidney	$21.1x10^4 \pm 0.6x10^4$	$15.6x10^4 \pm 1.0x10^4$	$26.2x10^4 \pm 2.3x10^4$	$9.2x10^4 \pm 3.9x10^4$ **
MRV	$0.3x10^4 \pm 0.1x10^4$	$0.3x10^4 \pm 0.2x10^4$	$2.6x10^4 \pm 1.1x10^4$	$0.05x10^4 \pm 0.01x10^4$
DRG	$0.7x10^4 \pm 0.4x10^4$	$0.5x10^4 \pm 0.3x10^4$	$0.8x10^4 \pm 0.2x10^4$	$0.3x10^4 \pm 0.3x10^4$

Figure 5.1 Effect of Ang II (1.1mg/kg/day for 14 days) compared with vehicle (saline) on eNOS mRNA expression in WT and αCGRP KO mice. eNOS mRNA expression in the (a) aorta and (b) heart, kidney, mesenteric resistance vessels and dorsal root ganglia, measured by RT-qPCR, expressed as copies/µl and normalised to SDHA, HPRT-1 and PLA_2. *p<0.05, **p<0.01 and ***p<0.001 compared to vehicle treated animals and ###p<0.001 compared to Ang II treated WTs. N=3-4.

5.9.2 The investigation into the effect of vehicle or Angiotensin II infusion for 14 days on Endothelin-1 (ET-1) protein and gene expression in WT and αCGRP KO mice, measured by ELISA and RT-qPCR.

The measurement of ET-1 was assessed in mixed gender WT and αCGRP KO mice after 14 days infusion of either Vehicle (saline) or Ang II. ET-1 mRNA expression was measured in the (a) aorta and (c) heart, kidney, mesenteric resistance vessels and dorsal root ganglia and expressed as copies/µl. Plasma samples were collected and purified in SEP-Columns to allow the extraction of plasma peptides. Once purified, samples were assayed for ET-1 by an enzyme linked-immunosorbent assay and expressed as pg/ml as shown in figure 5.2b. In the aorta, ET-1 mRNA expression was lowered after Ang II infusion in comparison to the vehicle treated animals. However statistical evaluation only showed significant downregulation of this mRNA expression in WTs and not CGRP KOs ($p<0.05$ Figure 5.2a). When observing the circulating plasma ET-1 concentration, statistical analysis by 2-way ANOVA + Bonferroni's post t-test showed no significantly different ET-1 concentration (pg/ml) in each of the treatment groups (Figure 5.2b). This could perhaps be due to the time frame of the experiment, in which perhaps 14 days was not long enough for plasma ET-1 concentrations to be increased in the circulation. With regards to the kidney, MRV and DRG, there was no change in mRNA expression in both WT and αCGRP KOs in the onset of hypertension (Figure 5.2c). However in the heart, ET-1 mRNA expression is raised in both WT and αCGRP KO mice in the onset of hypertension, this being significantly higher in the αCGRP KOs when compared to both their matched vehicle controls ($p<0.05$) and the hypertensive WTs ($p<0.01$). However it is also worth noting here that when comparing the vehicle treated animals, ET-1 expression in the heart is significantly higher in KO's compared to WTs ($p<0.05$, Figure 5.2c).



(c)

Organ	WT Veh	WT Ang II	KO Veh	KO Ang II
Heart	31.6±6.9	84.8±10.6	107±8.0 #	177.1±18.0 * ##
Kidney	80.4±18.9	39.3±10.7	62.9±28.1	80.8±41.4
MRV	$0.9x10^3±0.8x10^3$	$0.1x10^3±0.09x10^3$	$0.09x10^3±0.03x10^3$	$1.0x10^3±0.7x10^3$
DRG	$2.1x10^3±0.08x10^3$	$2.1x10^3±0.4x10^3$	$1.1x10^3±1.1x10^3$	$1.6x10^3±1.3x10^3$

Figure 5.2 Effect of Ang II (1.1mg/kg/day for 14 days) compared with vehicle (saline) on ET-1 mRNA and plasma protein expression in WT and αCGRP KO mice. ET-1 mRNA expression in the (a) aorta and (c) heart, kidney, mesenteric resistance vessels and dorsal root ganglia, measured by RT-qPCR, expressed as copies/µl and normalised to SDHA, HPRT-1 and PLA_2. (b) Representative circulating plasma ET-1 expression, expressed as pg/ml where *p<0.05 compared to vehicle treated animals, #p<0.05 compared to vehicle treated WTs and ##p<0.01 compared to Ang II treated WTs. N=3-4

5.9.3 Investigating the effect of Angiotensin II infusion for 14 days on VCAM-1 mRNA and protein expression in vascular tissues of WT and αCGRP KO mice by RT-qPCR and Immunohistochemistry.

Protein VCAM-1 localisation and expression in the aorta (Figure 5.3a-e) was determined by Immunohistochemistry and quantified and expressed as the % positive expression of the whole aortic section measured as shown in Figure 5.3f. Figures b-e are representative of positive staining for VCAM-1 in WT and αCGRP KO mice treated with either vehicle or Ang II compared to the negative control (a). Positive VCAM-1 expression was found in all vascular cells of the aorta (Figure 5.3b-e). When quantified, with regards to vehicle treated infusion, there was no difference in VCAM-1 expression between WT and αCGRP KO mice. VCAM-1 expression was significantly upregulated in the onset of hypertension in both WT ($p<0.001$) and αCGRP KO mice ($p<0.001$), however this expression was significantly higher in hypertensive αCGRP KO mice compared to WT Ang II infused mice ($p<0.001$, Figure 5.3f).

VCAM-1 mRNA expression was determined by RT-qPCR in the aorta in WT and αCGRP KO mice after 14 days of vehicle or Ang II infusion as shown in Figure 5.3g. Again there was no difference between the vehicle treated animals. In the onset of hypertension, mRNA expression showed similar trends to the protein expression determined by immunohistochemistry. VCAM-1 expression was again significantly upregulated in hypertensive WT ($p<0.01$) and αCGRP KO mice ($p<0.001$), with this expression being significantly higher again in hypertensive αCGRP KOs when compared to Ang II treated WTs (Figure 5.3g $p<0.01$).

VCAM-1 mRNA expression was then determined by RT-qPCR in the heart, kidney, MRV and DRG in WT and αCGRP KO mice after 14 days of vehicle or Ang II infusion as shown in Figure 5.4. With regards to the heart, MRV and DRG, no differences in VCAM-1 mRNA expression were found in WT and αCGRP KO mice after either vehicle or Ang II infusion. However in the kidney, VCAM-1 expression was significantly upregulated in the onset of hypertension in both WT ($p<0.05$) and αCGRP KO ($p<0.01$) mice after Ang II infusion. When comparing these two hypertensive groups, αCGRP KO mice had significantly more VCAM-1 mRNA expression when compared to the hypertensive WTs ($p<0.05$, Figure 5.4).

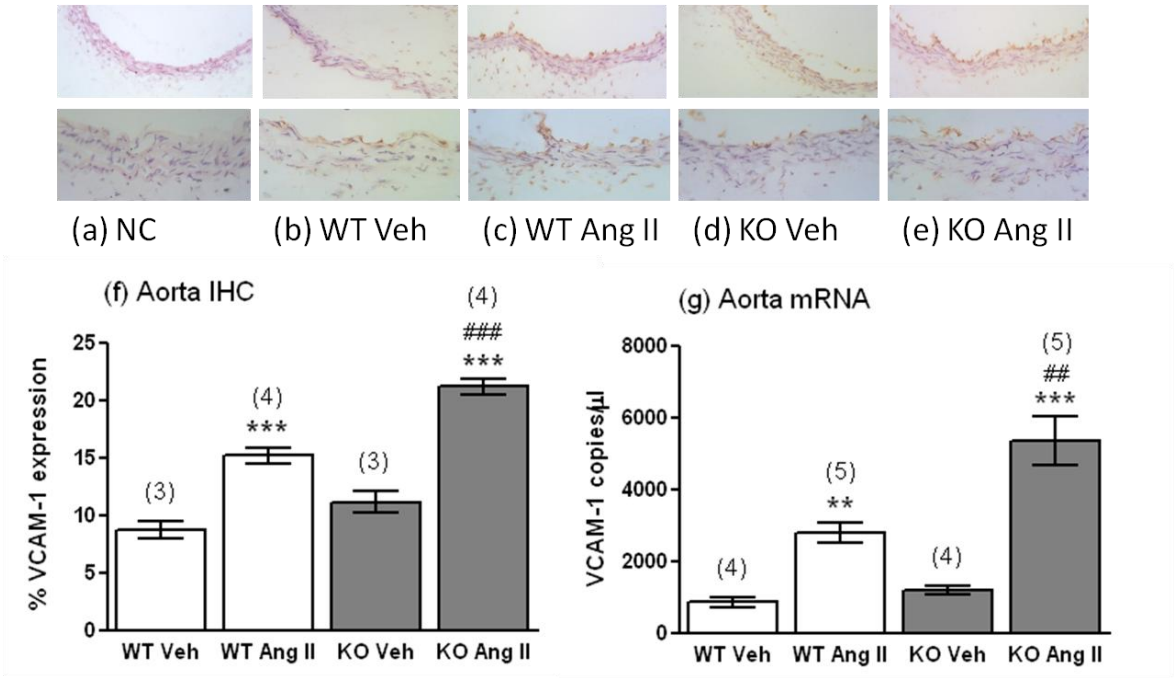


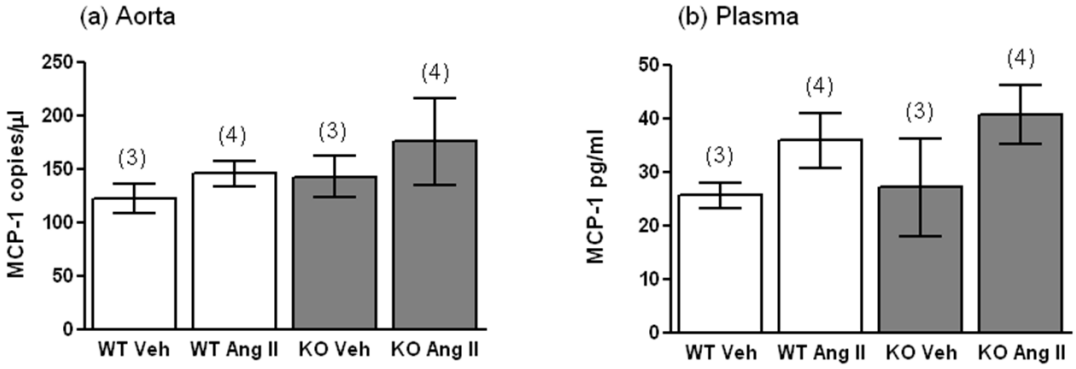
Figure 5.3 Effect of Ang II (1.1 mg/kg/day for 14 days) compared with vehicle (saline) on VCAM-1 mRNA and protein expression in WT and αCGRP KO mice. Immunostaining of VCAM-1 in the thoracic aorta (a) negative control (b) WT vehicle (c) WT Ang II (d) KO vehicle (e) KO Ang II at 40x magnification (top row) and 400x magnification (bottom row). Quantitative analysis of aortic VCAM protein expression (f) and representative mRNA expression by RT-qPCR measured by RT-qPCR, expressed as copies/µl and normalised to SDHA, HPRT-1 and PLA_2 (g). **p<0.01 and ***p<0.001 compared to vehicle. ##p<0.01 and ###p<0.001 compared to WT Ang II-treated animals. N=3-7

Organ	WT Veh	WT Ang II	KO Veh	KO Ang II
Heart	$5.6x10^3 \pm 2.0x10^3$	$7.8x10^3 \pm 0.6x10^3$	$7.8x10^3 \pm 1.9x10^3$	$7.4x10^3 \pm 2.2x10^3$
Kidney	$1.3x10^3 \pm 0.1x10^3$	$2.9x10^3 \pm 0.4x10^3$ *	$1.8x10^3 \pm 0.3x10^3$	$4.5x10^3 \pm 0.1x10^3$ ** #
MRV	$185.3x10^3 \pm 194.3x10^3$	$273.0x10^3 \pm 129.0x10^3$	$67.8x10^3 \pm 17.7x10^3$	$568.3x10^3 \pm 394.8x10^3$
DRG	$221.3x10^3 \pm 2.8x10^3$	$181.4x10^3 \pm 35x10^3$	$73.0x10^3 \pm 71.6x10^3$	$149.6x10^3 \pm 16.7x10^3$

Figure 5.4 Effect of Ang II (1.1mg/kg/day for 14 days) compared with vehicle (saline) on VCAM-1 mRNA expression in WT and αCGRP KO mice. VCAM-1 mRNA expression in the heart, kidney, mesenteric resistance vessels and dorsal root ganglia, measured by RT-qPCR, expressed as copies/µl and normalised to SDHA, HPRT-1 and PLA_2. *p<0.05 and **p<0.01 compared to vehicle treated groups and #P0.05 compared to Ang II treated WTs. WT Vehicle N=3, WT Ang II N=4, KO Vehicle N=3, KO Ang II N=4.

5.9.4 The investigation into the effect of vehicle or Angiotensin II infusion for 14 days on MCP-1 protein and gene expression in WT and αCGRP KO mice.

The measurement of MCP-1 was assessed in mixed gender WT and αCGRP KO mice after 14 days infusion of either Vehicle (saline) or Ang II. MCP-1 mRNA expression was measured in the (a) aorta and (c) heart, kidney, MRV and DRG and expressed as copies/µl. Plasma samples were assayed for MCP-1 by an ELISA and expressed as pg/ml as shown in figure 5.5b. In terms of mRNA expression, there was no difference in MCP-1 expression in both WT and αCGRP KO animals after vehicle (saline) infusion, and in the onset of hypertension in the aorta (Figure 5.5a), heart, kidney, MRV and DRG (Figure 5.5c). With regards to circulating plasma MCP-1, figure 5.5b indicates a trend towards an increase in MCP-1 expression in the onset of hypertension in WT and αCGRP KO mice, however statistical evaluation showed no significant difference between the treatment groups.



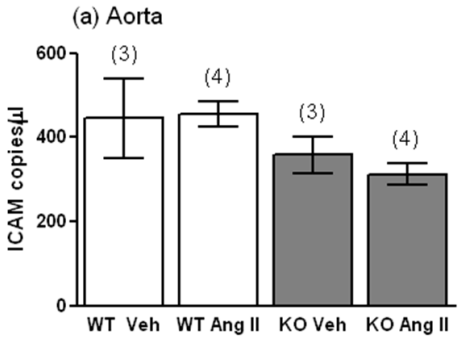
(c)

Organ	WT Veh	WT Ang II	KO Veh	KO Ang II
Heart	209.3±36.6	214.8±38.8	263±54.4	274.9±83.8
Kidney	196.7±19.3	183.1±35.8	84.7±26.3	127.5±30.7
MRV	$85.8x10^3±81.5x10^3$	$133.7x10^3±76.9x10^3$	$9.0x10^3±5.5x10^3$	$199.9x10^3±92.2x10^3$
DRG	$63.9x10^3±9.9x10^3$	$77.8x10^3±23.5x10^3$	$96.9x10^3±10.5x10^3$	$107.8x10^3±27.1x10^3$

Figure 5.5 Effect of Ang II (1.1mg/kg/day for 14 days) compared with vehicle (saline) on MCP-1 mRNA and plasma protein expression in WT and αCGRP KO mice. MCP-1 mRNA expression in the (a) aorta and (c) heart, kidney, mesenteric resistance vessels and dorsal root ganglia, measured by RT-qPCR, expressed as copies/µl and normalised to SDHA, HPRT-1 and PLA_2. (b) Representative circulating plasma MCP-1 expression, expressed as pg/ml. Statistical analysis by one-way ANOVA + Bonferroni's test showed no significant difference between the treatment groups N=3-4

5.9.5 Investigating the effect of Angiotensin II infusion for 14 days on ICAM-1 mRNA expression in vascular tissues of WT and αCGRP KO mice by RT-qPCR.

ICAM-1 mRNA expression as shown in Figure 5.6 was determined by RT-qPCR in the (a) aorta and (b) heart, kidney, MRV and DRG in WT and αCGRP KO mice after 14 days of vehicle or Ang II infusion. With regards to all tissues measured, there was no difference in ICAM-1 expression after saline infusion, or in the onset of hypertension. Copy numbers in the tissues are relatively low suggesting that either there is low ICAM-1 expression in these vascular tissues, or perhaps ICAM-1 may not be involved in this model of hypertension.



(b)

Organ	WT Veh	WT Ang II	KO Veh	KO Ang II
Heart	$0.5x10^3 \pm 0.07x10^3$	$0.8x10^3 \pm 0.09x10^3$	$0.6x10^3 \pm 0.77x10^3$	$0.6x10^3 \pm 0.09x10^3$
Kidney	$1.3x10^3 \pm 0.01x10^3$	$1.6x10^3 \pm 0.08x10^3$	$1.5x10^3 \pm 0.04x10^3$	$1.3x10^3 \pm x0.110^3$
MRV	$221.1x10^4 \pm 211.1x10^4$	$395.2x10^4 \pm 198.5x10^4$	$18.4x10^4 \pm 7.6x10^4$	$93.9x10^4 \pm 37.6x10^4$
DRG	$210.0x10^3 \pm 8.0x10^3$	$188.2x10^3 \pm 20.0x10^3$	$56.7x10^3 \pm 56.7x10^3$	$149.6x10^3 \pm 31.4x10^3$

Figure 5.6 Effect of Ang II (1.1mg/kg/day for 14 days) compared with vehicle (saline) on ICAM-1 mRNA expression in WT and αCGRP KO mice. ICAM-1 mRNA expression in the (a) aorta and (b) heart, kidney, mesenteric resistance vessels and dorsal root ganglia, measured by RT-qPCR, expressed as copies/µl and normalised to SDHA, HPRT-1 and PLA_2. Statistical analysis by one-way ANOVA + Bonferroni's test showed no significant difference between the treatment groups, N=3-4.

5.9.6 Investigating the effect of Angiotensin II infusion for 14 days on circulating plasma Interleukin 1, 10, 12 and KC expression in WT and αCGRP KO mice.

Circulating plasma interleukin-1, 10, 12 and KC expression as shown in Figure 5.7 was determined by immunoassay, described previously in chapter 2 materials and methods in WT and αCGRP KO mice after 14 days of vehicle or Ang II infusion and expressed as pg/ml. With regards to vehicle treatment and cytokine expression, there is no significant difference between WT and αCGRP KO mice after saline infusion for 14 days. IL-1 expression was increased in both WT and αCGRP KO mice in the onset of hypertension, this only being statistically significant in the αCGRP KO mice ($p<0.01$ Figure 5.7a), In terms of circulating KC (mouse IL-8 analogue) and IL-10, no differences were observed after the onset of Ang II induced hypertension in WT and αCGRP KO mice (Figure 5.7 b and c). Figure 5.7d shows representative plasma IL-12 expression in WT and αCGRP KO mice after vehicle or Ang II infusion for 14 days. When comparing the groups, IL-12 expression is significantly increased in both WT ($p<0.01$) and αCGRP KO ($p<0.001$) mice in the onset of hypertension, however there is no difference in IL-12 expression in the absence of CGRP during hypertension.

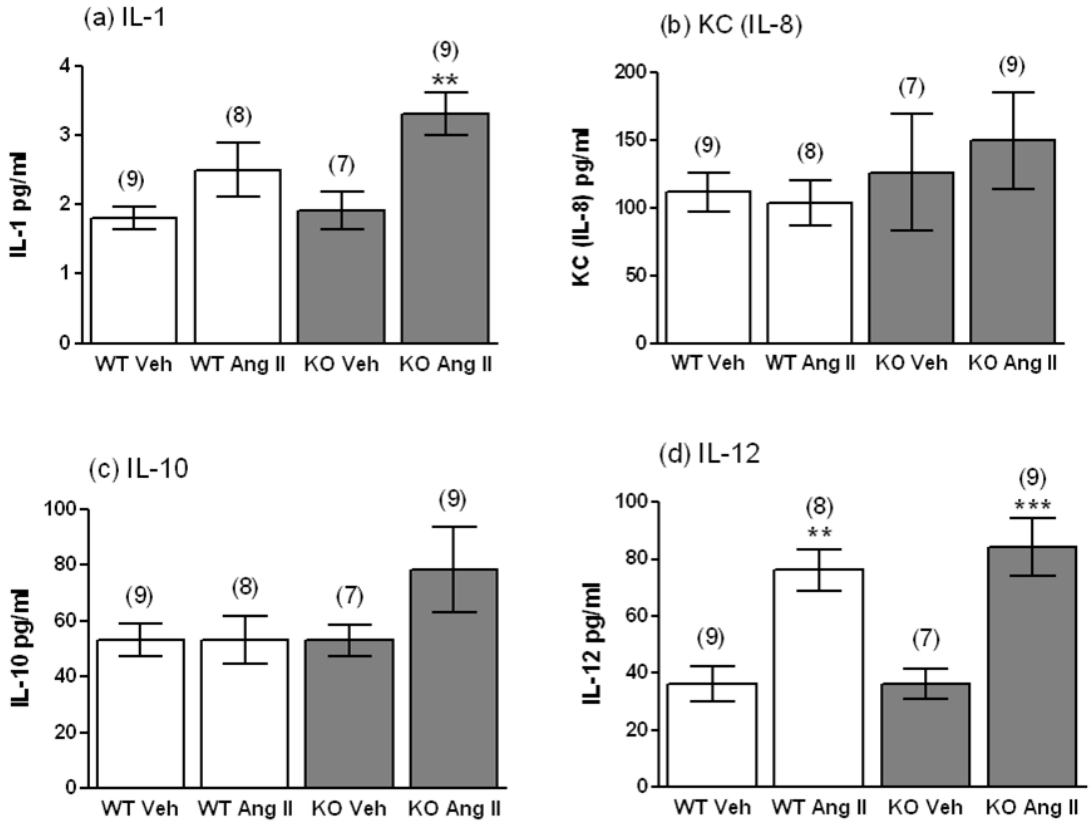
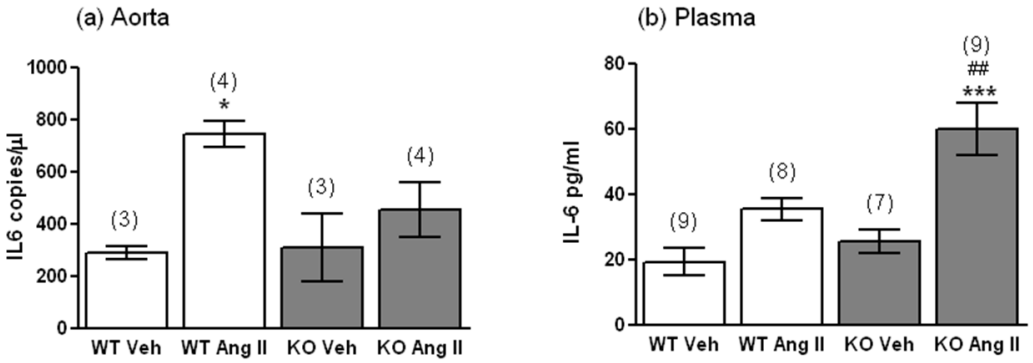


Figure 5.7 Effect of Ang II (1.1mg/kg/day for 14 days) compared with vehicle (saline) on circulating plasma Interleukin 1, 8, 10 and 12 expression in WT and αCGRP KO mice. Circulating plasma (a) IL1, (b) KC, (c) IL10 and (d) IL12 expression, expressed as pg/ml where **p<0.01 and ***p<0.001 compared to vehicle treated animals. N=7-9.

5.9.7 Investigating the effect of Angiotensin II infusion for 14 days on Interleukin 6 (IL-6) mRNA expression in vascular tissues and circulating plasma protein expression in WT and αCGRP KO mice.

IL-6 mRNA expression as shown in Figure 5.8 was determined by RT-qPCR in the aorta (figure 5.8a), heart, kidney, MRV and DRG (figure 5.8c) in WT and αCGRP KO mice after 14 days of vehicle or Ang II infusion. Circulating plasma protein IL-6 (figure 5.8b) was also determined by ELISA. In the aorta, no differences were observed between vehicle treated WT and αCGRP KO mice. IL-6 mRNA expression was significantly upregulated in WT mice in the onset of hypertension ($p<0.05$), however this increase was not observed in the αCGRP KO mice (Figure 5.8a). Circulating plasma IL-6 concentrations did not differ between saline control infused WT and αCGRP KO mice, however in the onset of hypertension, IL-6 was raised in WT mice (not significant) and αCGRP KO mice ($p<0.001$). This elevation of IL-6 in αCGRP KO mice was significantly higher in the absence of αCGRP when compared to Ang II treated WTs ($p<0.01$, Figure 5.8b). In the heart, MRV and DRG, no differences in IL-6 mRNA expression were observed in both vehicle treated WT and αCGRP KO mice, or in the onset of hypertension (Figure 5.8c). However in the kidney, IL-6 mRNA expression was significantly elevated in αCGRP KO mice ($p<0.05$), but not WT mice in the onset of hypertension. When comparing the two hypertensive groups, deletion of αCGRP caused significant upregulation of IL-6 mRNA in hearts of αCGRP KO mice compared to WTs after Ang II infusion for 14 days ($p<0.01$, Figure 5.8c).



(c)

Organ	WT Veh	WT Ang II	KO Veh	KO Ang II
Heart	$0.2x10^3±0.1x10^3$	$1.0x10^3±0.4x10^3$	$1.6x10^3±0.08x10^3$	$5.3x10^3±1.5x10^3$
Kidney	$0.1x10^3±0.02x10^3$	$0.1x10^3±0.004x10^3$	$0.6x10^3±0.1x10^3$	$2.0x10^3±0.4x10^3$ * ##
MRV	$8.6x10^3±4.9x10^3$	$19.4x10^3±10.0x10^3$	$8.8x10^3±2.0x10^3$	$27.9x10^3±21.2x10^3$
DRG	$5.1x10^3±0.6x10^3$	$6.1x10^3±1.6x10^3$	$8.4x10^3±1.2x10^3$	$5.9x10^3±1.0x10^3$

Figure 5.8 Effect of Ang II (1.1mg/kg/day for 14 days) compared with vehicle (saline) on IL6 mRNA and plasma protein expression in WT and αCGRP KO mice. IL6 mRNA expression in the (a) aorta and (c) heart, kidney, mesenteric resistance vessels and dorsal root ganglia, measured by RT-qPCR, expressed as copies/µl and normalised to SDHA, HPRT-1 and PLA_2. (b) Representative circulating plasma IL-6 expression, expressed as pg/ml where *p<0.05 and ***p<0.001 compared to vehicle treated animals and ##p<0.01 compared to Ang II treated WTs. mRNA N=4-5 and plasma N=7-9.

5.9.8 Investigating the effect of Angiotensin II infusion for 14 days on circulating TNF-α expression in plasma of WT and αCGRP KO mice by ELISA.

Circulating TNF-α levels as shown in Figure 5.9 was determined by ELISA in the plasma of WT and αCGRP KO mice after 14 days of vehicle or Ang II infusion and expressed as pg/ml. With regards to all treatment groups, there was no difference in TNF-α levels. It is worth noting that the concentration values are low. This could be due to the time frame of the experiment, in which perhaps 14 days was not long enough for plasma TNF-α concentration to be increased in the circulation.

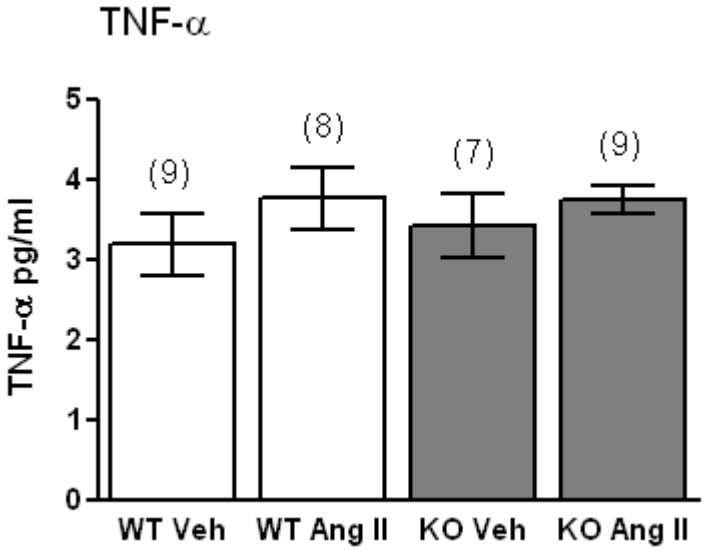


Figure 5.9 Effect of Ang II (1.1mg/kg/day for 14 days) compared with vehicle (saline) on circulating plasma TNF-α expression in WT and αCGRP KO mice. Circulating plasma TNF-α expression, expressed as pg/ml. Statistical evaluation by one-way ANOVA + Bonferroni's pot test showed no difference between the treatment groups. N=7-9.

5.9.9 Investigating the impact of either vehicle or Angiotensin II infusion for 14 days on vascular hypertrophy of the aorta and collagen expression in WT and αCGRP KO mice

Sections of thoracic aorta were collected from animals on day 14 and fixed and prepared in paraffin blocks before being sectioned and fixed onto polylysine slides for staining as described previously in chapter two. After staining with Masson's Trichrome stain, cross sections of aorta were examined and measured under a microscope to determine whether or not the hypertensive mice in this study exhibited symptoms of vascular remodelling shown in figure 5.10. I also wanted to determine whether there was any difference between the hypertensive αCGRP KO mice compared to their matched WTs. In order to assess this, both the aortic wall area and aortic wall width were measured, figure 5.11. An increase in aortic wall area indicates a widening of the vessel in response to increased pressure exerted upon the vessel wall, whereas an increased aortic wall width indicates a thickening of the vessel wall in response to the increased pressure placed upon it. Together, both are markers of vascular remodelling, a process usually associated with hypertension.

Analysis of mean aortic width measurements (μm) from Masson's Trichrome stained aortic sections in figure 5.11(a) indicate that the mean aortic wall width is significantly increased in WT and αCGRP KO mice infused with Ang II compared to their Vehicle treated counterparts. ($p<0.01$ and $p<0.001$, 2 way ANOVA and Bonferroni's test). When split to look at SMC 5.11(b) and Collagen 5.11(c) individually, figure 5.11(b) shows that mean SMC width (μm) is significantly increased in WT and αCGRP KO mice infused with Ang II compared to the Vehicle treated groups. ($p<0.05$, $p<0.01$ and $p<0.001$, 2 way ANOVA and Bonferroni's test). Figure c illustrates the differences in mean collagen width (μm). Collagen width is significantly increased in αCGRP KO mice compared to their vehicle treated counterparts ($p<0.05$, 2 way ANOVA and Bonferroni's).

Analysis of mean aortic area measurements (mm^2) from Masson's Trichrome stained aortic sections in figure 5.12(a) indicate that the mean aortic wall area is significantly increased in αCGRP KO mice infused with Ang II compared to both their Vehicle treated counterparts and Ang II infused WT mice. ($p<0.01$ and $p<0.001$, 2 way ANOVA and Bonferroni's test).

When looking at SMC 5.12(b) and collagen 5.12(c) individually, figure 5.12(b) shows that mean SMC area (mm^2) is significantly increased in αCGRP KO mice compared to both its vehicle counterparts and Ang II infused WT mice. ($p<0.5$ and $p<0.01$, 2 way ANOVA and Bonferroni's test). Figure c illustrates the differences in mean collagen area (mm^2). Collagen width is also significantly increased in αCGRP KO mice compared to their vehicle treated counterparts and Ang II infused WT mice, ($p<0.05$ and $p<0.001$, 2 way ANOVA and Bonferroni's post t-test).

Figure 5.13 illustrates collagen III mRNA expression in the (a) aorta and (b) heart, kidneys, MRV and DRG in WT and αCGRP KO mice treated with either saline (vehicle) or Ang II for 14 days. mRNA expression in these tissue were measured by RT-qPCR and results are expressed as copies/µl of cDNA. With regards to the heart, kidney, mesenteric resistance vessels and dorsal root ganglia as shown in figure 5.13b, collagen III mRNA expression did not differ between WT and αCGRP KO mice treated with the vehicle control, or in the onset of hypertension (Figure 5.13b). In the aorta there was no difference in collagen expression in vehicle treated WT and αCGRP KOs. In the onset of hypertension, collagen III expression was significantly elevated in αCGRP KO mice ($p<0.001$) but not WT mice compared to their matched vehicle controls. When comparing the two hypertensive groups together, collagen III expression was significantly upregulated in the absence of αCGRP when compared to Ang II infused WT mice ($p<0.01$, Figure 5.13a).

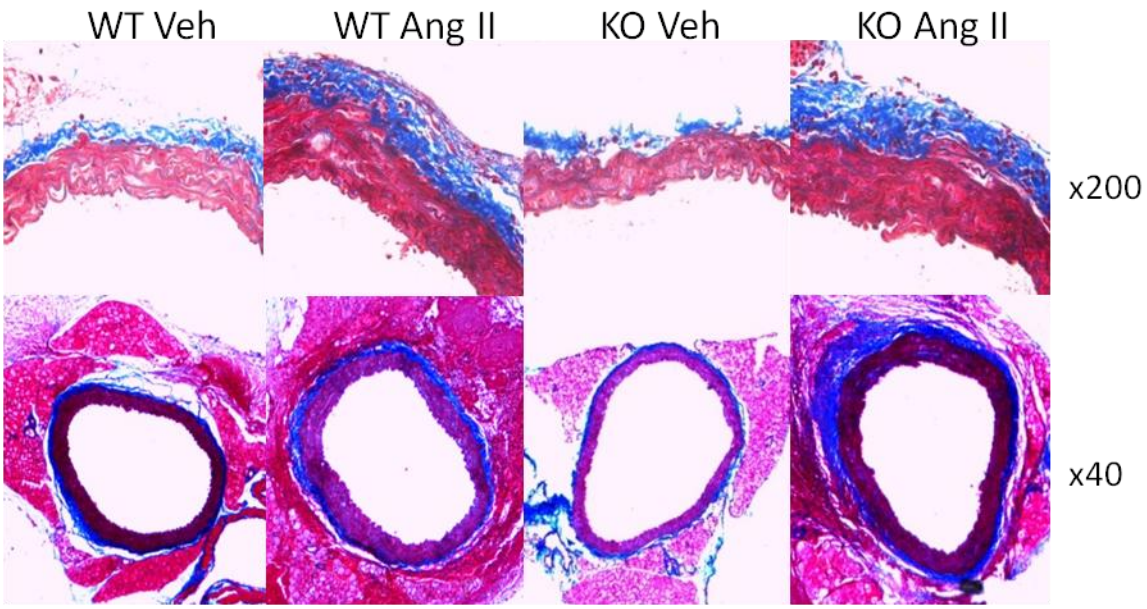


Figure 5.10 The effect of either vehicle or Angiotensin II infusion for 14 days on the aortic wall remodelling of WT and αCGRP KO mice. Showing representative images of aortic wall stained with Masson's trichrome at 40x and 200x magnification.

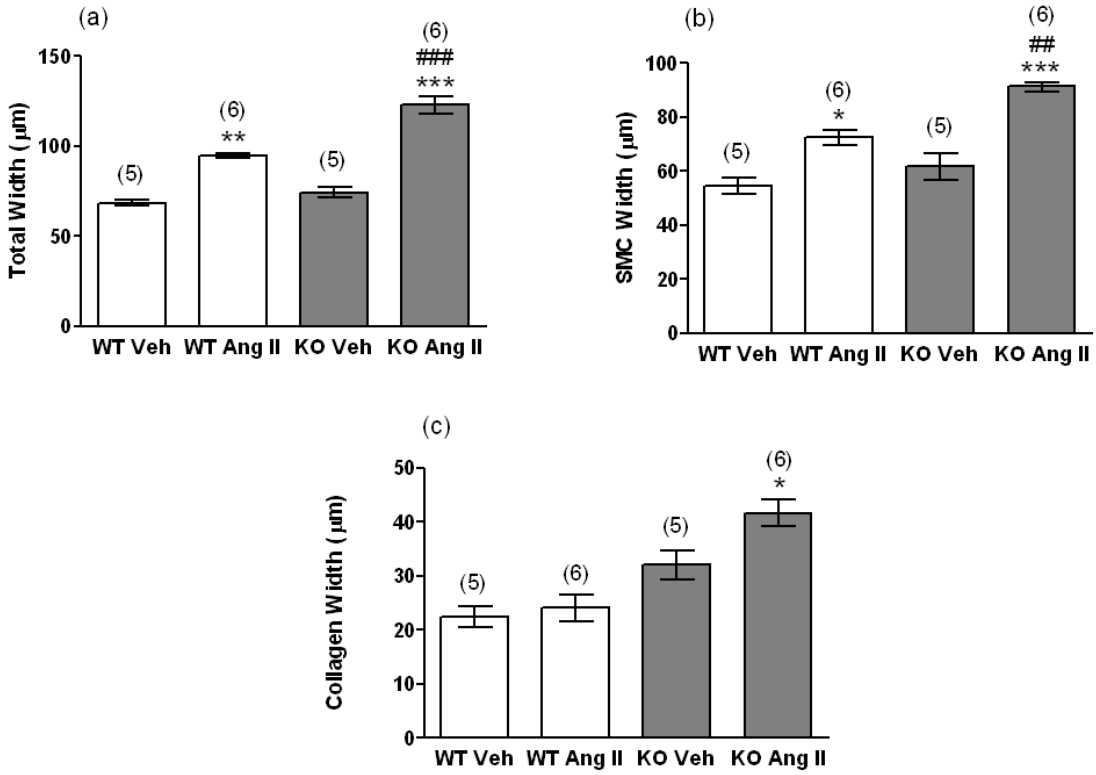


Figure 5.11 Total, SMC and collagen width (µm), measured from aortic sections taken from mixed gender WT and αCGRP KO mice treated with either Vehicle (saline) or Ang II infusion for 14 days. (a) Total width (SMC + collagen) in WT and αCGRP KO mice, (n=5-6). (b) Total SMC width (µm) in WT and αCGRP KO mice, (n=5-6) and (c) Total collagen width (µm) in WT and αCGRP KO mice, (n=5-6). Statistical evaluation of mean ± SEM by 2 way ANOVA and Bonferroni's test, where *=p<0.05, **=p<0.01 and ***=p<0.001. ##=p<0.01 and ###=p<0.001 when comparing the Ang II treated groups.

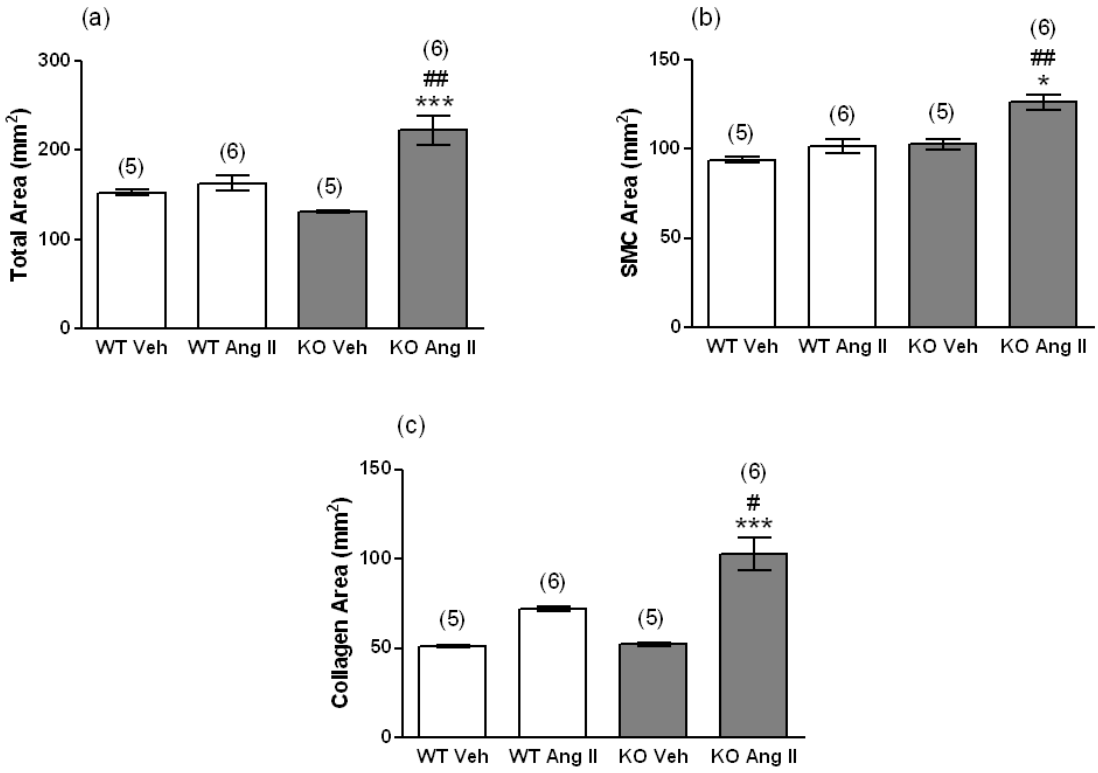
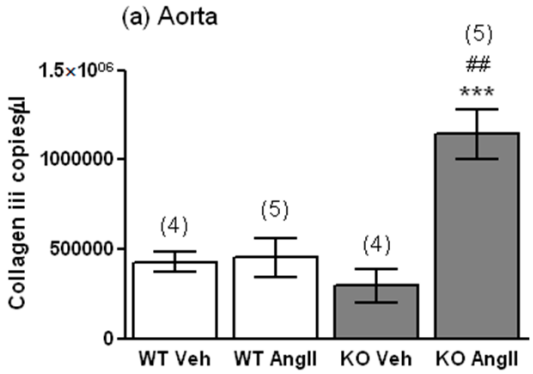


Figure 5.12 Total, SMC and collagen area (mm²) of mixed gender WT and αCGRP KO mice treated with either Vehicle (saline) or Ang II infusion for 14 days. (a) Total area (SMC + collagen) in WT and αCGRP KO, (n=5-6). (b) Total SMC area (mm²) in WT and αCGRP KO, (n=5-6) and (c) Total collagen area (mm²) in WT and αCGRP KO, (n=5-6). Statistical evaluation of mean ± SEM by 2 way ANOVA and Bonferroni's test, where *=$p<0.05$, and ***=$p<0.01$. #=$p<0.05$ and ##=$p<0.01$ when comparing the Ang II treated groups.



(b)

Organ	WT Veh	WT Ang II	KO Veh	KO Ang II
Heart	$29.2x10^3±7.5x10^3$	$26.2x10^3±4.3x10^3$	$24.1x10^3±0.6x10^3$	$32.7x10^3±3.6x10^3$
Kidney	$7.8x10^3±3.5x10^3$	$13.7x10^3±6.0x10^3$	$8.1x10^3±2.6x10^3$	$9.0x10^3±1.6x10^3$
MRV	$133.7x10^3±63.2x10^3$	$28.7x10^3±14.3x10^3$	$286.2x10^3±141.7x10^3$	$24.8x10^3±13.6x10^3$
DRG	$49.5x10^3±4.4x10^3$	$52.1x10^3±19.3x10^3$	$45.5x10^3±12.5x10^3$	$37.2x10^3±13.4x10^3$

Figure 5.13 Effect of Ang II (1.1mg/kg/day for 14 days) compared with vehicle (saline) on collagen III mRNA expression in WT and αCGRP KO mice. Collagen III mRNA expression in the (a) aorta and (b) heart, kidney, mesenteric resistance vessels and dorsal root ganglia, measured by RT-qPCR, expressed as copies/µl and normalised to SDHA, HPRT-1 and PLA_2. ***p<0.001 compared to vehicle treated animals and ##p<0.01 compared to Ang II treated WTs. N=4-5.

5.10 Summary

- eNOS mRNA expression is downregulated in the aorta in the onset of hypertension with αCGRP KOs showing an increased response to this eNOS downregulation after Ang II infusion. eNOS is reduced in hearts and kidneys of hypertensive mice with this reduction being significant in αCGRP KO mice compared to vehicle treated controls.

- ET-1 mRNA expression is reduced in the aorta in hypertension. This is significant in WTs but not αCGRP KOs. There is no difference in circulating plasma ET-concentrations after Ang II infusion. No change in mRNA expression was observed in the kidney, MRV and DRG in WT and αCGRP KOs in the onset of hypertension. In the heart, ET-1 mRNA expression is raised in both WT and αCGRP KO mice after Ang II, this being significantly more in the αCGRP KOs.

- VCAM-1 expression was found in all vascular cells of the aorta. There was no difference in VCAM-1 expression between vehicle treated WT and αCGRP KO mice. Aortic VCAM-1 protein expression was significantly upregulated in the onset of hypertension in both WT and αCGRP KO mice, this being significantly higher in hypertensive αCGRP KO mice compared to WTs. Aortic VCAM-1 mRNA expression was significantly upregulated in hypertension with this expression being significantly higher again in hypertensive αCGRP KOs. No differences in VCAM-1 mRNA expression were found in the heart, MRV and DRG after Ang II infusion. In the kidney, VCAM-1 expression was significantly upregulated in the onset of hypertension, this being significantly higher in αCGRP KOs compared to WTs.

- mRNA and protein MCP-1 expression did not differ in plasma and vascular tissue taken from WT and αCGRP KO mice treated with either vehicle (saline) or Ang II for 14 days.

- ICAM-1 mRNA expression did not differ in vascular tissue taken from WT and αCGRP KO mice after vehicle or Ang II infusion for 14 days.

- Plasma IL-1 expression was increased in both WT and CGRP KO mice in the onset of hypertension, this only being statistically significant in the αCGRP KO mice.

- No differences were observed in circulating plasma KC (mouse IL-8 analogue) and IL-10 expression after the onset of Ang II induced hypertension in WT and αCGRP KO mice .

- Plasma IL-12 expression is significantly increased in both WT and αCGRP KO mice in the onset of hypertension, however there is no difference in IL-12 expression in the absence of CGRP during hypertension.

- IL-6 mRNA expression was significantly increased in aortas of WT but not αCGRP KO mice in the onset of hypertension. Plasma IL-6 expression was raised in WT mice (not significant) and αCGRP KO mice after Ang II infusion. This elevation of IL-6 in αCGRP KO mice was significantly higher in the absence of αCGRP when compared to Ang II treated WTs. No differences in IL-6 mRNA expression were observed in the heart, MRV and DRG. In the kidney, IL-6 mRNA expression was significantly elevated in hypertensive αCGRP KOs, but not WTs. When comparing the two hypertensive groups, deletion of αCGRP caused significant upregulation of IL-6 mRNA in hearts of αCGRP KOs compared to WTs.

- No differences were observed in circulating plasma TNF-α expression after the onset of Ang II induced hypertension in WT and αCGRP KO mice.

- Vascular hypertrophy was apparent in the aortas of WT and αCGRP KO mice after Ang II infusion for 14 days. αCGRP KO mice displayed

exacerbated vascular hypertrophy in comparison to WTs, characterized by increased collagen deposition and mRNA expression.

5.11 Discussion

5.11.1 Stage 1- CGRP and Vasoactive mediators (ET-1 and eNOS) in Ang II induced hypertension

Endothelin-1 (ET-1) is a peptide secreted by vascular endothelial cells and acts as a potent vasoconstrictor. Its overall action is to maintain BP and vascular tone (Agapitov and Haynes, 2002), and as such, ET-1 levels have been shown to be elevated in hypertension when over-expressed in response to mediators such as Ang II. However, literature also shows inconsistent levels of ET-1 in hypertension, with either little or no effect (Hynen and Khalil, 2006: Schiffrin, 1995). During hypertension ET-1 acts on its receptors to induce vascular hypertension, proliferation and hypertrophy through increased oxidative stress and stimulation of pro-inflammatory markers (Duerrschmidt *et al.* 2000; Luft *et al.* 1999; Cheng *et al.* 2005; Schiffrin, 2001). The connection between Ang II and ET-1 is contradictory with studies reporting increased ET-1 expression in Ang II stimulated VSMCs of rats, but not in hypertensive salt-sensitive mREN2 rats (Rajagopalan *et al.* 1997; Moreau *et al.* 1997; Rossi *et al.* 2000). However in other models of hypertension including DOCA-salt (Larivière *et al.* 1993; Schiffrin *et al.* 1995), L-NAME (Verhagen *et al.* 1998) and the 2K1C-induced hypertension models (Sventek *et al.* 1996), ET-1 is elevated. ET-1 levels have also been shown to be elevated in urine samples, (Modesti *et al.* 2000).

CGRP has previously been proposed to play a beneficial counterbalancing role in several experimental models of hypertension characterized by an upregulated ET-system (Supowit *et al.* 1997; Wang *et al.* 2004; Xie *et al.* 2009). CGRP and ET-1 levels have both been previously reported to be increased in rats with cirrhotic cardiomyopathy (CCM, Fu *et al.* 2001) and in patients with portal hypertension formation (Zhan *et al*. 1999). Also, in patients with congestive heart failure, CGRP is decreased, whereas ET-1 levels are increased compared to healthy control patients. However when these patients are treated with nitroglycerin, CGRP in turn causes a reduction in ET-1 expression, therefore an improvement in the cardiovascular health of the patient occurs (Yu *et al.* 2003). Exogenous CGRP has previously been shown to dissociate complexes between ET-1 and ET_A receptors and relax VSMC through increased cAMP production, thus enhancing

vasodilatation. However, these anti-ET-1 effects of CGRP are not successfully mimicked by other vasodilators which cause release of endothelium-derived relaxing factors (acetylcholine) or stimulate NO release (sodium nitroprusside, Meens *et al.* 2010). It is thought that CGRP inhibits ET-1 effects through involvement of the G protein βγ subunits (Gβγ) as shown recently in isolated arteries from rat treated with Gallein (Gβγ-inhibitor, Meens et al. 2011).

In the present study we showed ET-1 mRNA expression to be reduced in the aorta in Ang II induced hypertension. This was significant in WTs but not αCGRP KOs. When we assayed for plasma ET-1 however, there was no difference in circulating ET-concentrations after Ang II infusion. There was also no change in ET-1 mRNA expression in the kidney, MRV and DRG in WT and αCGRP KOs in the onset of hypertension. However, in the heart, ET-1 mRNA expression is raised in both WT and αCGRP KO mice after Ang II, this being significantly more in the αCGRP KOs, which we would expect due to the significant increase in BP and lack of the vasodilation by CGRP in response to increasing Ang II levels. The inconsistent findings in regards to circulating plasma ET-1 levels in hypertension are not surprising. This is due to knowledge that elimination of ET-1 from the bloodstream occurs rapidly. Also the time limit of 14 days in this present study may not be long enough for circulating levels to be elevated in the circulation. With this in mind, it deemed sensible to measure ET-1 levels in the vascular tissue. However the differing results also found in the tissue may indicate a tissue specific role for ET-1 in the onset of Ang II induced hypertension. Therefore results identifying an interactive role for CGRP and ET-1 production in the onset of hypertension in this model are inconclusive.

As previously mentioned in the introduction, eNOS generates NO via L-arginine to regulate vascular tone (BP control) and pressure under normal physiological conditions. eNOS production activation is increased in response to shear stress and increased vasoconstrictor agents such as ET-1 and abnormalities in this endothelial production of NO result in endothelial dysfunction and the progression of atherosclerosis, vascular remodelling and hypertension (Moncada *et al.* 1993). eNOS mutant mice have been shown to have a hypertensive phenotype under baseline conditions (Huang *et al.* 1995) and inhibition of eNOS in Apo-E-deficient mice using double knockout mice results in significant aortic atheroma formation

and elevated BP (Knowles *et al.* 2000). On the other hand, increased eNOS expression has been shown to reduce hypertension and hyperinsulinemia in fructose-treated rats via increased NO bioavailability and vascular relaxation (Zhao *et al.* 2010). In addition to this gene transfer of human eNOS improved endothelial function and reduced hypertension in spontaneously hypertensive rats (Alexander *et al.* 2000). These studies highlight the potential of using eNOS gene therapy in the treatment of hypertension.

In the present study we found mRNA expression to be downregulated in the aorta in the onset of hypertension with αCGRP KOs showing an increased response to this eNOS downregulation after Ang II infusion. eNOS was also reduced in hearts and kidneys of hypertensive mice with this reduction again being significant in αCGRP KO mice compared to vehicle treated controls. This therefore indicates that the onset of endothelial dysfunction is prominent in response to the increase in BP by Ang II infusion. In addition to this, the further reduction in eNOS expression in the αCGRP KOs indicates a higher degree of endothelial dysfunction within these animals, which also fits in with the exacerbated hypertension found in the absence of αCGRP. This result suggests a role for CGRP in the regulation of eNOS expression in the onset of Ang II induced hypertension. CGRP release has been shown previously to promote endothelial cell proliferation and activate eNOS via the cAMP-PKA pathway in angiogenesis (Haegerstrand *et al.* 1990; Zheng *et al.* 2010). The vascular interaction between nitric oxide (NO) and CGRP was studied by Lee *et al.* using WT and eNOS KO mice treated with the NO donor nitroglycerin (NTG). In this study they showed that NTG induced significant release of CGRP in WT but not eNOS KO mice, therefore indicating that NTG-induced CGRP release is eNOS dependent. This study was gender specific as this result was not found in males, and the results give light to possible cardiovascular protection in females through CGRP and eNOS interaction (Lee *et al.* 2003). It is also possible that the enhanced loss of eNOS expression is secondary to the raised hypertension observed in αCGRP KOs, compared with WT mice.

5.11.2 Stage 2- CGRP, Adhesion Molecules and Inflammatory Cytokines in Angiotensin II induced hypertension

In order to elucidate mechanisms behind the protective role for CGRP in Ang II induced hypertension, the inflammatory profile of these mice were investigated. A range of important pro-inflammatory markers which have previously been shown to be implicated in Ang II induced hypertension and vascular inflammation were assessed. The results are now discussed in turn.

IL-1

Plasma IL-1 expression was enhanced in the onset of hypertension, this being significantly so in the αCGRP KO mice. This potentially fits in with previous reports whereby Il-1 has been shown to enhance the proliferation of VSMCs and fibroblasts *in vitro* (Thalmann and Meier, 2007) and also enhance the Ang II mediated effects via upregulation of the AT_1 receptor expression on cultured rat VSMCs (Sasamura *et al.* 1997). IL-1 production has also been shown to be increased in the renal cortex of rats in the Ang II-high salt model of hypertension (Ang II at 65ng/min subcutaneously and 8% NaCl diet for 14 days,). This increase in production was accompanied by an elevation in BP and the onset of vascular remodelling and renal damage (Boesen *et al.* 2008). This research fits in with ours as we have seen observed increased hypertrophy and vascular remodelling of the aorta in the onset of Ang II induced hypertension at day 14, and this may potentially be mediated through an increase in IL-1 expression.

IL-6

In this study we have shown IL-6 expression to be elevated in plasma and some vascular tissues (kidney and aorta) of mice in the onset of hypertension. In the plasma and kidney, this elevation appeared to be exacerbated in the absence of αCGRP; however this was not the case in the aorta. It is not known why this is the case, but may be that the role is tissue specific. It has been documented previously that plasma levels of IL-6 correlate with high BP and Ang II is thought to be a stimulator of IL-6 production from various cell types (Zhang *et al.* 2012;

Lee *et al.* 2005). In a high dose Ang II or high-salt diet model of hypertension, it was shown that C57BL/6 male WT and interleukin-6 KO (IL-6 KO) mice did not have any difference in BP recordings at baseline and MAP did not change significantly when placed on the high salt diet (4% NaCl). However when infused with high dose Ang II (90ng/min), MAP increased rapidly in both WT and IL-6 KO mice, but this increase plateaued in the KO mice at day 2, whilst the MAP in WTs continued to rise until the end of the 14 days where there was a ~30mmHg difference between the WT and KO mice. This suggests that IL-6 plays an important role in contributing to Ang II induced hypertension and independently of renal injury due to the rapid early response to Ang II and not later on when vessel remodelling and organ damage occurs (Lee *et al.* 2005). In addition to this, IL-6 protein expression has been shown to be elevated in patients with chronic kidney disease (CKD), however these levels are elevated further in CKD patients with hypertension, therefore suggesting that Ang II is a causative factor responsible for IL-6 induction in the kidney (Zhang *et al.* 2012). In the same study they also demonstrated that IL-6 deletion in the C57BL/6 mouse significantly reduced hypertension and renal damage after Ang II infusion compared to WT mice. The results shown in this chapter fit in with that documented in the literature, and it is possible that the enhanced IL-6 expression in the αCGRP KO mouse is dependent on the increased BP alone, and not via a direct role with the loss of αCGRP in this model.

KC and IL-10

No differences were observed in circulating plasma KC (mouse IL-8 analogue) and IL-10 expression after the onset of Ang II induced hypertension in WT and αCGRP KO mice, therefore suggesting that IL-8 and IL-10 do not play a functional role in this model. However IL-8 has previously been shown to be upregulated in cultured VSMCs from the thoracic aorta of the SHR compared to normotensive Wister rats under basal conditions. Ang II infusion to these rats elevates IL-8 expression; however this is significantly more elevated in the SHRs (Kim *et al.* 2008). Enhanced plasma IL-8 levels have also been detected in the rat after 4 hours Ang II treatment (Nabah *et al.*2004). We have previously shown within our group that KC expression in the kidney was elevated in the hypertensive C57BL/6

WT mice fed a high fat diet for 12 weeks. This study also reported elevated IL-10 levels in hypertensive WT mice fed a high fat diet (Marshall *et al.* 2009), IL-10 is an anti-inflammatory cytokine which can suppress the production of pro-inflammatory mediators such as IL-12 (Sutterwala and Mosser, 1999), IL-6 and 8 (Shimauchi *et al.* 1999) and TNFα *in vivo* (Clarke *et al.* 1998). With regard to Ang II, IL-10 has been shown to counteract the impaired endothelial-dependent relaxation induced by Ang II in aortic rings from C57BL/6 mice (Zemse *et al.* 2007). It is unknown at this stage as to why our data does not fit with that previously published, however we can conclude that the absence of CGRP does not affect the expression and regulation of these cytokines in this model at this timepoint.

IL-12

Ang II stimulates IL-12 production in cultured human dendritic cells (Wei-guo *et al.* 2010). This cytokine has also been shown by RT-qPCR to be enhanced in the aortas of ApoE-/- mice treated with Ang II for 14 days (Bruemmer *et al.* 2003). These studies suggest a role for IL-12 in the onset of Ang II induced inflammation, and support our findings whereby we found plasma IL-12 expression to be significantly increased in both WT and αCGRP KO mice in the onset of Ang II induced hypertension. With regards to CGRP, αCGRP and βCGRP have been reported previously to equally suppress the production of IL-12 and TNFα in bone marrow-derived dendritic cells stimulated with LPS from C57BL/6 WT mice. However this inhibition was not observed in the cells from RAMP1 KO mice, therefore suggesting that CGRP signalling through CLR/RAMP1 receptors plays a pivotal role in the regulation of pro-inflammatory cytokine production (Tsujikawa *et al.* 2007). We report no difference in IL-12 expression when comparing the hypertensive WTs to the hypertensive αCGRP KOs in our study. The reason for this may perhaps be due to a compensatory role played by βCGRP in these KO mice to inhibit the IL-12 elevation.

VCAM-1, MCP-1 and ICAM-1

The adhesion molecules ICAM-1 and VCAM-1 are widely documented to be upregulated by Ang II (Cheng *et al.* 2005). It has been shown *in vitro* that Ang II enhances ICAM and VCAM-1 expression in cultured human VSMC and endothelial cells (Pueyo *et al.* 2000; Pastore *et al.* 1999). In a rat model of high human renin hypertension, VCAM-1 and ICAM-1 expression was found to be increased and located in the interstitium, intima and adventitia of the small renal vessels (Mervaala *et al.* 1999). Male Wistar rats infused with Ang II for 6 days develop hypertension and exhibit marked increases in aortic VCAM-1 mRNA and protein expression (Tummala *et al*. 1999).

The chemokine MCP-1 is also reported to be induced in endothelial cells, monocytes and VSMCs in response to Ang II (Savoia and Schiffrin, 2007). Inhibition of MCP-1 by transfecting a deletion mutant into the rat resulted in a suppressed response to Ang II induced hypertension, characterised by destabilization of atherosclerotic lesions (Ni *et al.* 2004). This blockade of MCP-1 also enhances the survival rate in mice with myocardial infarction (Hayashidani *et al.* 2003.

However these mediators are not always enhanced in this model, and may perhaps be tissue/species/cell type specific. Ang II stimulated rat aortic cultured cells display increased VCAM-1, MCP-1 and ICAM-1 expression by RT-qPCR (Toba *et al.* 2005) however Ang II treatment had no effect on VCAM-1 and ICAM-1 expression in human cardiac microvascular endothelial cells (Grafe *et al.* 1997).

With regards to the role of CGRP and the expression of these markers, MCP-1 expression has been shown previously to be inhibited by CGRP transfection into isolated rabbit jugular vein grafts with the chronic inflammatory vein graft disease (Zhang *et al.* 2010). In addition to this, gene transfer of CGRP into Lewis rats suppressed VCAM-1 mRNA expression in the development of allograft vasculopathy (Zhang *et al.* 2009). These studies suggest an anti-inflammatory role for CGRP.

In a hypertension study using the αCGRP/calcitonin KO by Bowers *et al*. VCAM-1, MCP-1 and ICAM-1 expression in the kidney was measured by Immunohistochemistry in WT and αCGRP/calcitonin KO mice after either 14 or 21

days DOCA-salt treatment to induce hypertension. At baseline there was no difference in expression of these markers between the genotypes. These markers were elevated in the onset of hypertension at day 14, and were progressively more elevated at day 21. However when comparing the genotypes, the KO's had progressively elevated levels of these inflammatory markers compared to the WTs (Bowers *et al.* 2005). In this study we have also shown exacerbated VCAM-1 expression in the kidneys of hypertensive αCGRP KO mice, which supports the findings by Bowers *et al.* We also found this elevation to be exacerbated in the aorta, thus suggesting that αCGRP is protective in the onset of vascular inflammation. However our results for ICAM-1 and MCP-1 do not support that of Bowers *et al.* whereby we observed no difference in expression between the groups either before or after Ang II infusion, suggesting that these markers do not play a role in our particular model at this time at least.

TNFα

It is surprising that TNFα expression was not elevated in this model of hypertension, despite previous reports suggesting that ACE inhibitors suppress TNFα expression and synthesis both *in vivo* and *in vitro* (Fukuzawa *et al.* 1997). Plasma TNFα expression is enhanced in hypertensive patients when compared to normotensive controls (Dorffel *et al.* 1999). Ruiz-Ortega and co workers have also reported renal TNFα expression to be elevated in rats after Ang II infusion for 3 days (Ruiz-Ortega *et al.* 2002). It is possible that circulating TNFα is not increased at this time-point of the hypertension but tissue TNFα expression is, therefore a possible point for future investigation. We would have expected however for this plasma expression to be increased due to the increased IL-6 expression and the understanding that TNFα stimulates IL-6 production from monocytes (Ruiz-Ortega *et al.* 2002), but this was not the case in this study.

5.11.3 Stage 3- CGRP and Vascular Hypertrophy in Angiotensin II induced hypertension

The proliferation of vascular smooth muscle cells is a critical pathophysiological event in many cardiovascular diseases, such as hypertension and atherosclerosis. Hyperplasia or hypertrophy of vascular smooth muscle cells is mainly responsible for this vascular remodelling, which contributes to the elevated BP observed in hypertension. Angiotensin is not only involved in the regulation of vascular tone, but also participates in the mediation of cell growth and extracellular matrix deposition. In the present study angiotensin II induced the proliferation of vascular smooth muscle cells, characterised by the increase in lumen width and area in hypertensive mice, suggesting that angiotensin II is an important contributor to vascular smooth muscle cell proliferation. This has been reported previously, (Duff *et al.* 1992; Liao *et al.* 1996).

Vascular hypertrophy is considered to be an adaptive response to increased arterial wall stress in hypertension, (Obayashi *et al*.1999). It is well documented that Ang II induces vascular remodelling through both direct effects of increased BP and indirectly through vascular inflammation. In the vasculature, Ang II binds to the AT1 receptor which leads to the activation of oxidative stress, mainly mediated by reduced NADPH oxidase. Inflammation and oxidative stress seems to be closely correlated and may mediate vascular remodelling, (Marchesi *et al.* 2008).

Studies have found that endogenous CGRP is involved in the depressor effect and regression of vascular remodelling of losartan or perindopril in reno-hypertensive rats, (Qin *et al*. 2004). Previous findings have also shown that CGRP inhibited the proliferation of vascular smooth muscle cells from rabbit and rat aorta induced by fetal bovine serum through an increase in cAMP production (Li *et al.* 1997). Intramuscular gene transfer of CGRP was also shown to inhibit neointimal hyperplasia after balloon injury in the abdominal aorta of the rat through inhibition of VSMC and CGRP-mediated apoptosis (Wang and Wang, 2004). Prior to this study, Wang and Wang also showed CGRP to inhibit rat VSMC proliferation via a NO and CGRP interaction (Wang and Wang, 1999).

In a study by Li *et al.* (2010), isoprenaline (beta-adrenergic agonist) was given to rats as a cardiac remodelling model. This infusion was shown to significantly

increase left ventricle to body weight ratio in response to cardiac apoptosis and collagen deposition, alongside a reduction in CGRP production. This reduction in CGRP however was reversed by the administration of the TRP channel activator Rutaecarpine, therefore suggesting that Rutaecarpine was able to reverse isoprenaline-induced cardiac remodelling through stimulating CGRP production (Li *et al.* 2010). This association was also previously shown in renovascular hypertensive rats whereby CGRP release via Rutaecarpine treatment reduced BP and the progression of vascular remodelling in the 2K1C induced model of hypertension (Qin *et al.* 2007). Also sensory CGRP depletion by chronic capsaicin treatment has been reported to induce exacerbated hypoxia-induced hypertension and vascular remodelling in rats (Tjen *et al.* 1998).

In our model of Ang II-induced hypertension, with regards to vascular hypertrophy, significant results were identified in the aorta. As expected, infusion with Ang II for 14 days caused vascular hypertrophy of the thoracic aorta in both WT and αCGRP KO mice. Both smooth muscle cell width (μm) and area (mm^2) was increased in Ang II treated groups. It was also clear that smooth muscle cell width and area was significantly increased in αCGRP KO mice compared to WT mice infused with Ang II. In addition to this the αCGRP KO mice developed significantly enhanced amounts of collagen deposition surrounding the aortic lumen, which was observed both by morphological analysis and when analysing aortic mRNA expression of collagen III. These results support the hypothesis that CGRP has an inhibitory effect on the proliferation of vascular smooth muscle cells and plays a protective role in Ang II induced hypertension. However, the mechanism underlying this protective effect of CGRP on vascular smooth muscle cell proliferation is unclear. CGRP perhaps may have reduced the viability and DNA synthesis of vascular smooth muscle cells, and thus decreased the increased proliferation index of vascular smooth muscle cells and collagen formation induced by Ang II. It has been reported that CGRP inhibits the proliferation of vascular smooth muscle cells in conjunction with an elevation of the second-messenger, cAMP (Li *et al.* 1997). It is also well documented that ERK1/2, the important protein kinases in cell proliferation, are activated by many growth factors via different upstream signal proteins. It has been reported that angiotensin II activates extracellular signal-regulated kinase (ERK1/2) via protein kinase C-zeta (PKC-ζ) by cross-talk with the tyrosine kinase receptor pathway,

(Liao *et al.* 1997). CGRP was recently shown to inhibit both Ang II-induced and hypoxia-induced proliferation of rat aortic and pulmonary smooth muscle cells via inactivation of the extracellular signal-regulated protein kinase 1/2 (ERK1/2), therefore suggesting that the inhibitory effect of CGRP on the proliferation of vascular smooth muscle cells involves the MAPK signalling pathway (Qin *et al.* 2004; Li *et al.* 2012).

CHAPTER SIX: THE EFFECTS OF ANGIOTENSIN II INFUSION FOR 14 DAYS ON REACTIVE OXYGEN SPECIES PRODUCTION, MARKERS OF OXIDATIVE STRESS AND THE ANTIOXIDANT DEFENSE SYSTEM IN WT AND αCGRP KO MICE

6.1 Introduction

Antioxidants and reactive oxygen species (ROS) are defined as chemically reactive molecules which have an important role in cell signalling and maintaining homeostasis within the vasculature (Irani, 2001). However in response to stress stimuli, excess production and accumulation of ROS leads to a state of oxidative stress causing detrimental effects to the tissue (Lassegue and Griendling, 2004). The cells therefore require a robust antioxidant defence mechanism in order to combat against the increasing oxidative stress. These two mechanisms and their role in hypertension are now discussed in turn.

6.2 The antioxidant defence system

As previously mentioned in the general introduction, under normal physiological conditions, ROS levels are strictly modulated by the antioxidant defence system, present in all living organisms and evolved to protect the cells and combat oxidative stress. Low antioxidant bioavailability also promotes the onset of oxidative stress. During the oxidative stress pathway, oxidation occurs when an electron is transferred from a substance to an oxidising agent to produce free radicals and cause cell damage, and in some cases cell death. Antioxidants are molecules which are capable of inhibiting the oxidation of other molecules. They also terminate the oxidative reaction chain by removing free radicals by oxidising themselves, therefore making them reducing agents. The antioxidant defence system is comprised of enzymatic ROS scavengers; including superoxide dismutase (SOD), catalase, glutathione peroxidase (GPX) and haem oxygenase (HO-1) and non enzymatic ROS scavengers such as glutathione, urate and vitamins (Zadak *et al.* 2009). Evidence of the role of antioxidants in protecting against the onset of hypertension is conflicting, with some studies reporting the benefits of antioxidant supplements in clinical hypertension, while others do not

(Sedeek *et al.* 2003; Paravicini and Touyz, 2008; Touyz and Briones, 2011). Despite these results, an effective antioxidant defence mechanism is required in order to defend against the Ang II induced ROS production and end point tissue damage. In this study we wanted to identify the state of the oxidant defence system in our model and therefore we decided to measure the expression of a number of antioxidant genes by RT-qPCR which now will be discussed in turn.

6.2.1 Heme oxygenase-1 (HO1)

Heme oxygenase (HO) is an enzyme which catalyzes the degradation of heme to produce biliverdin, iron and carbon monoxide, and the HO pathway has been described as the most powerful protective antioxidant mechanism against oxidative stress (Maines, 1988). HO-1 mechanism is induced by oxidative stress, therefore this gene can be used as both an indicator of ROS production and as a marker of antioxidant defence. There are 2 known isoforms of heme oxygenase, HO-1 being an inducible form and HO-2; a constitutive form. For the purpose of this thesis we focused on HO-1 as it is known to respond primarily to stress such as oxidative stress, hypoxia and cytokines (Hedlund *et al.* 1997). HO-1 is found in almost all cardiovascular tissues including VSMCs, endothelial cells and cardiomyocytes (Christodoulides *et al.* 1995; Morita *et al.* 1995; Eytssen-Hernandez *et al.* 1996; Motterlini *et al.* 1995). Within the vessel wall it acts as a powerful antioxidant and modulates vessel tone and BP by producing carbon monoxide (CO, a vasodilator) and biliverdin which in turn increases cGMP due to activation of soluble guanylate cyclase and stimulates calcium activated potassium channels and also reduces NAPDH induced ROS production (Chen *et al.* 2003; Levere *et al*. 1990).

There is evidence to suggest a protective role for HO-1 in the onset of hypertension. In the SHR, HO-1 administration lowers BP in a dose-dependent manner (Levere *et al.* 1990; Martasek *et al.* 1991; Sabaawy *et al*. 2001). In the Ang II induced hypertension model, NADPH-dependent superoxide production is known to be present in the vessel wall, therefore causing damage. HO-1 has been shown to be upregulated in this model in order to potentially counteract the NADPH-dependent superoxide production provoked by Ang II and the damage caused. Ishizaka *et al.* (1997) have also showed both mRNA and protein HO-1

expression to be upregulated in the rat aorta after 7 days Ang II infusion (Ishizaka *et al.* 1997), and in the renal vasculature, HO-1 has also been shown to inhibit Ang II-induced superoxide production in mouse ascending loop of Henle (TALH) cells (Kelsen *et al.* 2008).

6.2.2 Glutathione peroxidase (GPX)

Another antioxidant system against oxidant damage is the glutathione redox cycle, which is composed of the enzymes glutathione peroxidase (GPX) and glutathione reductase (GSSG-Rd), and the co substrates NADPH and glutathione (De Cavanagh *et al.* 2000). Glutathione peroxidase-1 (GPX-1) is one of the most important antioxidant enzymes known to date and over expression delays endothelial cell growth and increases resistance to oxidative stress (Kiss *et al*. 1998). It functions as a scavenger for ROS and maintains its biological role by reducing lipid hydroperoxides to their corresponding alcohols in order to reduce free hydrogen peroxide to water. There are 8 isoforms (GPX 1-8), however GPX-1 is the most abundant form, being found in the cytoplasm of all mammalian tissues. GPX-1 has been recently suggested to play a positive role in antioxidant defence in the progression of hypertension. Raised glutathione-related antioxidant defence markers in blood were observed in elderly hypertensive patients. In hypertension, treatment with ACE inhibitors has been shown to improve endothelial dysfunction and delay the progression of atherosclerosis through oxidative stress inhibition and antioxidant elevation. Recent studies have suggested that the mechanisms by which these drugs do this are thought to be through upregulation of the glutathione-dependent antioxidant defences (De Cavanagh *et al.* 1999; De Cavanagh *et al.* 2000). Oxidative stress and BP is exacerbated in the GPX-1 deficient mouse compared to WT's (Gao *et al*. 2010). Overall these findings suggest a protective role for GPX-1 in combating against oxidative stress in the onset of hypertension and vascular related diseases.

6.2.3 Intracellular Superoxide Dismutase (SOD1)

The superoxide dismutases (SOD) are enzymes which catalyze the dismutation of superoxide into oxygen and hydrogen peroxide, therefore making them important

in the antioxidant defence in all cells exposed to oxygen (Garrido and Griendling, 2009). There are three forms of SOD in humans and mammals, named SOD1, SOD2 and SOD3. Intracellular SOD (SOD1) is the intracellular antioxidant form found in the cytoplasm and involved in inflammation which facilitates the conversion of superoxide to hydrogen peroxide for degradation. SOD2 is found in the mitochondria and SOD3 is extracellular. We will concentrate on the SOD1 form in this thesis. This powerful antioxidant has been studied in inflammatory conditions where treatment with SOD1 decreases ROS generation and oxidative stress through the inhibition of endothelial activation therefore making this antioxidant important in the potential for new therapies in inflammatory conditions (Segui *et al.* 2004). Studies using the SOD1 KO mouse in Ang II induced hypertension have identified a protective mechanism for SOD in the reducing BP and oxidative stress (Gongora *et al.* 2006). This SOD isoform plays a fundamental role in the control of vascular tone due to its ability to reduce NO inactivation by superoxide and produce the dilatory hydrogen peroxide, which may be important in the onset of hypertension (Fukai and Ushio-Fukai, 2011). Aortic rings from SOD1 KO mice also display impaired endothelial dependent relaxation compared to WT mice (Didion *et al*. 2002). In a mouse model of hypertension, elevated SOD1 expression and decreased superoxide production was reported previously in mice infused with Ang II (Wang *et al*. 2002).

6.3 Reactive oxygen species and the onset of oxidative stress

Sources of ROS within the vessel include uncoupled nitric oxide synthase, cytochrome P450, NADPH oxidases and xanthine oxidoreductase (Rivera *et al*. 2010). In this study we will focus on the NADPH oxidases as they are considered the main source of ROS. These will be discussed further in section 6.3.1. The ROS family include superoxide anions ($O^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($HO^{\cdot}$). When ROS formation occurs in response to external stimuli, it begins with the one electron reduction of molecular oxygen (O_2) which forms superoxide. This superoxide is then metabolised by SOD to form H_2O_2. At this point, the H_2O_2 is then metabolised into water by GPX (Paravicini and Touyz, 2006). This reaction is summarised in Figure 1.2 from the general introduction in chapter one. NO, a powerful antioxidant, is produced within the vessel wall and

plays a pivotal role in regulating vascular tone and BP in order to maintain redox balance. However under stress conditions such as the onset of hypertension, there is an overproduction of ROS, which outweighs the production of NO, causing a subsequent imbalance in the redox state, sometimes referred to a “redox shift”. This increase in ROS formation within the vessel wall causes cellular antioxidant defences to become overwhelmed and therefore unable to counteract the increase in ROS. This excess in ROS results in cellular dysfunction due to damage of lipids, proteins and DNA, which in turn is a key marker of the onset of cardiovascular disease. This elevation of ROS then contributes to the elevation of oxidative stress, vascular inflammation and endothelial dysfunction.

6.3.1 NADPH oxidases as a source of ROS in hypertension

The nicotinamide adenine dinucleotide phosphate reduced oxidases (NADPH oxidases) are a family of enzymes known to primarily produce ROS and be the major source of ROS in the vessel wall, with *in vitro* and *in vivo* studies previously showing that this ROS production can be inhibited using NADPH oxidase inhibitors such as apocynin and DPI (diphenyleneiodonium (Vlahos *et al.* 2010; Selemidis *et al.* 2008; Grimminger *et al.* 1995). As already mentioned, NADPH oxidase is an enzyme responsible for the source of ROS production in the innate immune response. This family of multimeric enzyme complexes generate superoxide production by the transfer of elections from NADPH to O_2. There are seven members of the NADPH family known to date, named NOX (1-5) and Duox (1-2), however only 4 of these are known to exist in the blood vessel wall (NOX1, 2, 4 and 5, found in endothelium, VSMC and adventitial fibroblasts (Van *et al.* 2008; Li and Shah, 2002; Chamseddine and Miller, 2003). All seven are encoded by separate genes and are largely varied in their individual amino acid sequence. Their tertiary structure is formed of a cytoplasmic N-terminus attached to a 6 transmembrane domain, containing four histidine residues forming two binding sites, and a long C-terminal cytosolic tail. NOX 5 and Duox 1 and 2 are fully functional isoforms on their own, however NOX1-4 require interaction with regulatory subunits, one of these being p22phox. NOX4 and its interaction with p22phox forms a fully functional protein, however NOX1-3 require additional regulatory peptides. These are; p67 phox which is the cytosolic activator subunit,

p47phox which directs the protein to the membrane, and finally a small GTPase (Rac1 or Rac2) which is required for translocation and activation (Manea, 2010; Lassegue and Griendling, 2009; Brandes *et al.* 2010). A substantial amount of research has been carried out to investigate the role of NADPH oxidases in cardiovascular disease and hypertension, however as mentioned in the general introduction, in this thesis I focussed on looking at the NOX 2 and NOX 4 isoforms.

NOX2 when activated produces extracellular superoxide which is known to cause damage to surrounding cells (Bedard and Krause, 2007) and this isoform has been commonly associated with the damage caused in the Ang II induced model of hypertension (Paravicini and Touyz, 2008). Hypertension and vascular hypertrophy has been shown to be exacerbated in NOX2 over expressing transgenic mice after Ang II infusion, in addition to increased superoxide production in the endothelium (Bendall *et al.* 2007). Overexpression of NOX2 in the aorta has also been shown to significantly worsen the hypertension (Lassegue and Griendling, 2010). Studies by Wang *et al.* and Carlstrom *et al.* indicated a pro-oxidative role for NOX 2 in the Ang II induced model of hypertension where NOX2 KO mice showed decreased constrictor responses and hence lower BP than WT Ang II infused mice under basal conditions. This was also accompanied by lower superoxide production and nitrotyrosine production (Wang *et al.* 2001; Carlstrom *et al*. 2009). NOX2 is also implemented in hypertrophy, this being independent of BP and hypertension. In the 2K1C model of hypertension which causes over reactivity of the RAS, AT_1 activation upregulates NOX2 in the left ventricle and results in increased superoxide production and cardiac hypertrophy (Wang *et al.* 2007). Also in studies by Johar *et al.* and Bendall *et al.* using NOX2 KO mice it was shown that Ang II activates NOX2 which mediates fibrosis in the heart and cardiac hypertrophy (Johar *et al.* 2006; Bendall *et al.* 2002).

Whilst the role of NOX2 in the Ang II induced model of hypertension is well documented, the role of NOX4 in hypertension is not so clear, perhaps mainly due to a lack of conclusive findings by which some report NOX 4 to have positive influences on vascular smooth muscle growth, while others do not (Lassegue and Griendling, 2010). NOX4 is found on intracellular membranes, abundantly expressed in the kidneys, but also found in the vascular cells, fibroblasts and cardiomyocytes (Lassegue and Griendling, 2010). As mentioned before, NOX4

differs from the other isoforms in that it predominantly generates hydrogen peroxide (Dikalov *et al.* 2008) which is known to be a vasodilator (Matoba and Shimokawa, 2003). Inconclusive findings have been reported in the Ang II induced model of hypertension where some studies have shown increased NOX4 mRNA expression in hypertensive WTs (Wingler *et al.*2001; Mollnau *et al.* 2002), however others have reported decreased NOX4 mRNA expression in WT mice (Lassegue *et al.* 2001). Ang II was also shown to decrease NOX4 expression in cultured VSMCs (Lassegue *et al.* 2001) and Chabrashvili *et al.* showed *in vivo* that Ang II infusion causes AT_1 dependent reduction of renal NOX4. NOX4 is also considered to have influences on Ang II pressor responses with endothelial NOX4 overexpressing mice displaying lower BP as a direct consequence of increased hydrogen peroxide (Ray *et al.* 2011). In the 2K1C model, Wang *et al.* showed AT_1 dependent upregulation of NOX4 in the heart and aortas of hypertensive mice displaying elevated BP and cardiac remodelling in comparison to their normotensive controls (Wang *et al.* 2007). A role for NOX4 in cardiac fibroblasts has also been suggested due to it being shown to correlate with TGF-β induced conversion of cardiomyocytes to myofibroblasts (Cucoranu *et al.* 2005).

6.4 Summary of the background

Sensory nerves are known to act via perivascular neuronal networks to release neuropeptides including CGRP. These neuropeptides work in combination with the autonomic nervous system to regulate physiological vascular tone and pathophysiological disease processes. Sensory nerve endings have been shown to be in contact with VSMCs and ECs, therefore ideally placed to influence both the physiological and pathophysiological control of the heart and vasculature, and also meaning that these nerves can be influenced by ROS signalling and inflammatory mechanisms. With this in mind, it is feasible to predict that due to the vasodilatory properties of CGRP in the onset of inflammatory conditions, this neuropeptide may perhaps play a role in protecting against ROS formation and hence oxidative stress and end point vascular damage.

6.5 Hypothesis

Under basal conditions, no difference in markers of oxidative stress will be observed between WT and αCGRP KO mice. However, based on the increased

BP and vascular hypertrophy shown in previous chapters, when infused with Ang II for 14 days, αCGRP KO mice will be more susceptible to increased markers of oxidative stress.

6.6 Aims

- Based on the literature of oxidative stress in the Ang II induced model of hypertension, the aim of this chapter is to investigate levels of oxidative stress using markers previously reported to be elevated in WT mice (HO-1, GPX, SOD1, NOX2, TGF-β and NOX4).

- To measure mRNA expression of HO-1, GPX, SOD1, NOX2, TGF-β and NOX4 in WT and αCGRP KO mice treated with either vehicle or Ang II for 14 days and observe and differences in the absence of αCGRP.

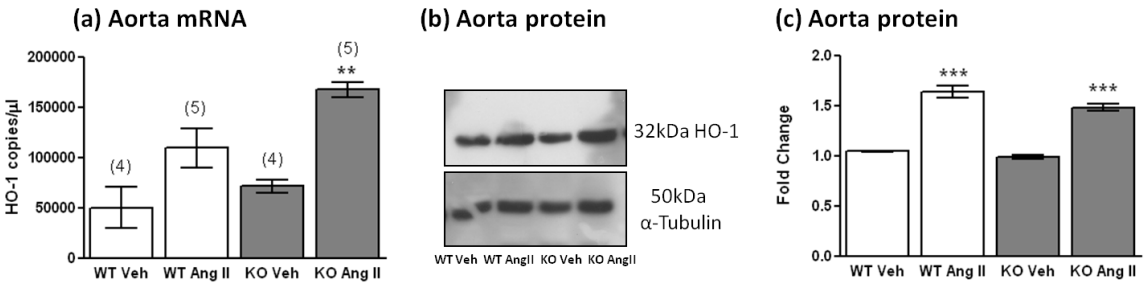
- To confirm HO-1 and NOX4 protein expression by Western Blotting and determine any differences between hypertensive WT and αCGRP KO mice.

- To localise NOX4 in the aorta using immunohistochemical techniques.

6.7 Results

6.7.1 Investigating the effect of Angiotensin II infusion for 14 days on Heme-Oxygenase 1 (HO-1) mRNA and protein expression in vascular tissues of WT and αCGRP KO mice.

HO-1 mRNA expression as shown in Figure 6.1 was determined by RT-qPCR in the aorta (figure 6.1a), heart, kidney, MRV and DRG (figure 6.1d) in WT and αCGRP KO mice after 14 days of vehicle or Ang II infusion. Protein HO-1 expression in the aorta (figure 6.1b and c) was also determined by Western blotting. With regards to vehicle treated animals, there was no difference in both mRNA and protein expression between WT and αCGRP KO's in all vascular tissue. In the aorta, HO-1 mRNA expression was upregulated with Ang II infusion in WT mice as expected. This upregulation was significant in αCGRP KO mice after Ang II infusion ($p<0.01$). Protein HO-1 expression was significantly upregulated in both WT and αCGRP KO mice after 14 days Ang II infusion (figure 6.1b and d). No changes were observed in both the heart and DRG, however in the kidney, hypertensive αCGRP KO mice displayed significant increases in HO-1 mRNA expression in comparison to their vehicle treated counterparts ($p<0.001$).



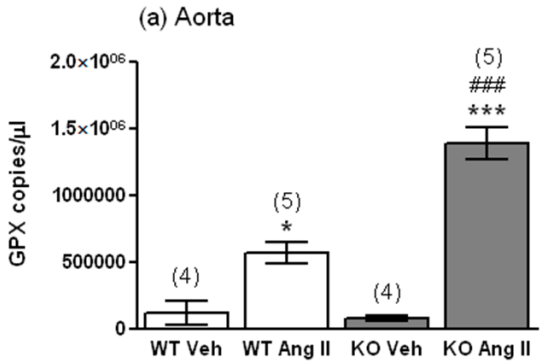
(d)

Organ	WT Veh	WT Ang II	KO Veh	KO Ang II
Heart	$8.9x10^4±3.6x10^4$	$9.3x10^4±3.2x10^4$	$9.1x10^4±5.8x10^4$	$9.1x10^4±0.4x10^4$
Kidney	$10.1x10^4±0.1x10^4$	$15.3x10^4±1.1x10^4$	$7.0x10^4±2.4x10^4$	$16.5x10^4 0.04±x10^4$ ***
MRV	$69.2x10^4±66.2x10^4$	$3.8x10^4±1.9x10^4$	$4.0x10^4±2.6x10^4$	$29.1x10^4±25.6x10^4$
DRG	$10.5x10^5±0.1x10^5$	$7.7x10^5±5.0x10^5$	$13.9x10^5±2.7x10^5$	$11.7x10^5±4.6x10^5$

Figure 6.1 Effect of Ang II (1.1mg/kg/day for 14 days) compared with vehicle (saline) on HO-1 mRNA and protein expression in WT and αCGRP KO mice. HO-1 mRNA expression in the aorta (a).Representative HO-1 protein expression in the mouse aorta (b) and protein HO-1, expressed as fold change relative to the matched vehicle control and normalised to α-tubulin (c). HO-1 mRNA expression in the heart, kidney, mesenteric resistance vessels and dorsal root ganglia, measured by RT-qPCR, expressed as copies/µl and normalised to SDHA, HPRT-1 and PLA_2. (d). **p<0.01 and ***p<0.001 compared to vehicle treated animals. N=4-5.

6.7.2 Investigating the effect of Angiotensin II infusion for 14 days on Glutathione Peroxidase-1 (GPX) mRNA expression in vascular tissues of WT and αCGRP KO mice.

GPX mRNA expression as shown in Figure 6.2 was determined by RT-qPCR in the (a) aorta and (b) heart, kidney, MRV and DRG in WT and αCGRP KO mice after 14 days of vehicle or Ang II infusion. With regards to vehicle treated animals, again no differences in mRNA expression were observed between WT and αCGRP KO mice in all tissues measured. In the aorta (6.2a) GPX mRNA expression was significantly increased in hypertensive WT ($p<0.05$) and αCGRP KO mice ($p<0.001$) after Ang II infusion. However αCGRP KOs were more susceptible to this increase in GPX compared to hypertensive WTs ($p<0.001$). In the heart, GPX mRNA expression was significantly upregulated again after Ang II infusion in WT ($p<0.05$) and αCGRP KO mice ($p<0.01$, 6.2b). However this expression in the heart was not significantly different between WT and αCGRP KO mice. No differences in GPX mRNA expression was observed between the 4 treatments groups in the kidney, MRV or DRG (Figure 6.2b).



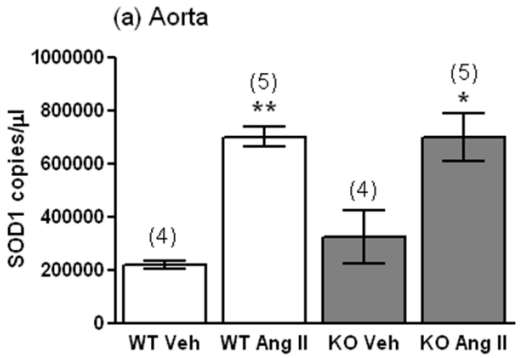
(b)

Organ	WT Veh	WT Ang II	KO Veh	KO Ang II
Heart	$2.2x10^5±0.05x10^5$	$3.2x10^5±0.2x10^5$ *	$2.0x10^5±0.2x10^5$	$4.0x10^5±0.1x10^5$ **
Kidney	$2.5x10^6±0.1x10^6$	$6.7x10^6±4.3x10^6$	$1.8x10^6±0.04x10^6$	$6.4x10^6±2.7x10^6$
MRV	$25x10^6±18.7x10^6$	$159x10^6±57.8x10^6$	$57.1x10^6±56.9x10^6$	$20.5x10^6±19.7x10^6$
DRG	$1.3x10^4±0.1x10^4$	$419.6x10^4±366.7x10^4$	$1.5x10^4±0.1x10^4$	$526.x10^4±477.x10^4$

Figure 6.2 Effect of Ang II (1.1mg/kg/day for 14 days) compared with vehicle (saline) on GPX mRNA expression in WT and αCGRP KO mice. GPX mRNA expression in the (a) aorta and (b) heart, kidney, mesenteric resistance vessels and dorsal root ganglia, measured by RT-qPCR, expressed as copies/µl and normalised to SDHA, HPRT-1 and PLA_2. *p<0.05, **p<0.01 and ***p<0.001 compared to vehicle treated animals and ###p<0.001 compared to Ang II treated WTs. N=4-5.

6.7.3 Investigating the effect of Angiotensin II infusion for 14 days on Superoxide Dismutase 1 (SOD1) mRNA expression in vascular tissues of WT and αCGRP KO mice.

SOD1 mRNA expression as shown in Figure 6.3 was determined by RT-qPCR in the (a) aorta and (b) heart, kidney, MRV and DRG in WT and αCGRP KO mice after 14 days of vehicle or Ang II infusion. With regards to vehicle treated animals, again no differences in SOD1 mRNA expression were observed between WT and αCGRP KO mice in all tissues measured. In the aorta (6.3a) SOD mRNA expression was significantly increased in hypertensive WT ($p<0.01$) and αCGRP KO mice ($p<0.05$), however there was no difference in SOD1 expression between the hypertensive groups. In the heart (6.3b), SOD1 mRNA expression was significantly upregulated again in hypertensive αCGRP KO mice ($p<0.01$) however this was not significantly different to hypertensive WT mice. With regards to the MRV, there are trends of increasing expression in hypertensive animals; however this is not statistically significant. There were no differences in SOD1 mRNA expression between the four treatment groups in the kidney and DRG (6.3b).



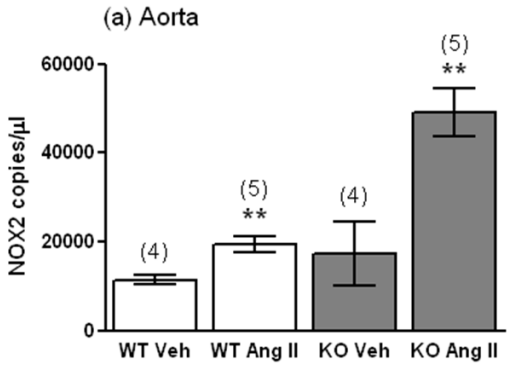
(b)

Organ	WT Veh	WT Ang II	KO Veh	KO Ang II
Heart	$2.7x10^6±0.5x10^6$	$4.7x10^6±0.3x10^6$	$1.3x10^6±0.9x10^6$	$6.2x10^6±0.5x10^6$ **
Kidney	$1.0x10^6±0.5x10^6$	$0.8x10^6±0.4x10^6$	$1.0x10^6±0.3x10^6$	$0.8x10^6±0.3x10^6$
MRV	$6.1x10^4±3.8x10^4$	$16.8x10^4±7.8x10^4$	$3.4x10^4±0.1x10^4$	$42.1x10^4±28.4x10^4$
DRG	$3.8x10^4±1.0x10^4$	$3.4x10^4±0.3x10^4$	$4.5x10^4±1.2x10^4$	$4.2x10^4±0.4x10^4$

Figure 6.3 Effect of Ang II (1.1mg/kg/day for 14 days) compared with vehicle (saline) on SOD1 mRNA expression in WT and αCGRP KO mice. SOD1 mRNA expression in the (a) aorta and (b) heart, kidney, mesenteric resistance vessels and dorsal root ganglia, measured by RT-qPCR, expressed as copies/µl and normalised to SDHA, HPRT-1 and PLA_2. *p<0.05, **p<0.01 and ***p<0.001 compared to vehicle treated animals. N=4-5.

6.7.4 Investigating the effect of Angiotensin II infusion for 14 days on NADPH-oxidase 2 (NOX2) mRNA expression in vascular tissues of WT and αCGRP KO mice.

NOX2 mRNA expression as shown in Figure 6.4 was determined by RT-qPCR in the aorta (figure 6.4a), heart, kidney, MRV and DRG (figure 6.4d) in WT and αCGRP KO mice after 14 days of vehicle or Ang II infusion. With regards to vehicle treated animals, there was no difference in both mRNA and protein expression between WT and αCGRP KO's in all vascular tissue. With regards to the WTs, mRNA NOX2 expression was upregulated in all tissue measured, however this was only significant in the aorta (figure 6.4d, $p<0.05$). In the αCGRP KO mice however, NOX2 mRNA expression was significantly upregulated after Ang II for 14 days in the aorta (figure 6.4a, $p<0.01$), heart ($p<0.01$) and kidney ($p<0.05$, figure 6.4d). In terms of differences between WT and αCGRP KO mice in the onset of hypertension, NOX2 mRNA expression was upregulated in the MRV and significantly upregulated in the hearts of αCGRP KO mice when compared to hypertensive WTs ($p<0.05$, figure 6.4d).



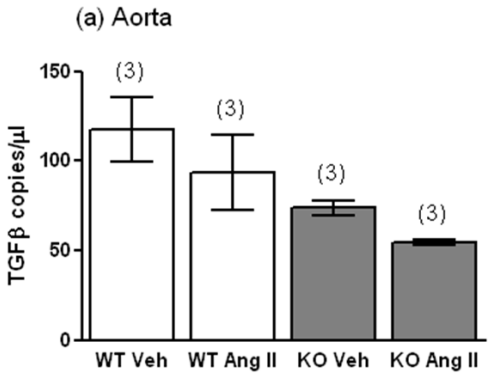
(b)

Organ	WT Veh	WT Ang II	KO Veh	KO Ang II
Heart	$43.2x10^3 \pm 4.9x10^3$	$55.0x10^3 \pm 3.4x10^3$	$43.0x10^3 \pm 7.2x10^3$	$91.4x10^3 \pm 11.1x10^3$ ** #
Kidney	$12.9x10^3 \pm 5.7x10^3$	$27.1x10^3 \pm 1.1x10^3$	$14.8x10^3 \pm 4.6x10^3$	$43.9x10^3 \pm 6.7x10^3$ *
MRV	$0.4x10^3 \pm 0.4x10^3$	$0.6x10^3 \pm 0.6x10^3$	$2.5x10^3 \pm 2.2x10^3$	$6.1x10^3 \pm 2.3x10^3$
DRG	$0.4x10^3 \pm 0.07x10^3$	$1.2x10^3 \pm 0.2x10^3$	$0.4x10^3 \pm 0.05x10^3$	$1.6x10^3 \pm 0.9x10^3$

Figure 6.4 Effect of Ang II (1.1mg/kg/day for 14 days) compared with vehicle (saline) on NOX2 mRNA expression in WT and αCGRP KO mice. NOX2 mRNA expression in the (a) aorta and (b) heart, kidney, mesenteric resistance vessels and dorsal root ganglia, measured by RT-qPCR, expressed as copies/µl and normalised to SDHA, HPRT-1 and PLA_2. *p<0.05, **p<0.01 and ***p<0.001 compared to vehicle treated animals. #p<0.05 compared to WT Ang II treated animals. N=4-5.

6.7.5 Investigating the effect of Angiotensin II infusion for 14 days on TGF-β mRNA expression in vascular tissues of WT and αCGRP KO mice.

TGF-β mRNA expression, as shown in Figure 6.5, was determined by RT-qPCR in the aorta (figure 6.5a), heart, kidney, MRV and DRG (figure 6.5b) in WT and αCGRP KO mice after 14 days of either vehicle or Ang II infusion. With regards to vehicle treated animals, there was no difference in TGF-β mRNA expression between WT and αCGRP KO's in all vascular tissue. In the aorta (a), heart, kidney and MRV (b) there was no difference in TGF-β mRNA expression in the onset of hypertension in both WT and αCGRP KO mice. When looking at expression in the DRG, TGF-β mRNA is elevated in both WT and αCGRP KO mice in the onset of hypertension ($p<0.01$), however this upregulation is similar in both genotypes, suggesting no role for αCGRP in influencing TGF-β expression in this model of hypertension (figure 6.5b).



(b)

Organ	WT Veh	WT Ang II	KO Veh	KO Ang II
Heart	22.5±2.9	16.6±2.7	28.0±2.4	19.6±3.6
Kidney	12.0±1.3	7.3±1.0	8.4±2.2	7.6±1.4
MRV	$3.8x10^3±3.8x10^3$	$2.2x10^3±1.1x10^3$	$0.02x10^3±0.02x10^3$	$57.7x10^3±52.5x10^3$
DRG	$4.5x10^3±0.5x10^3$	$11.1x10^3±0.4x10^3$ **	$9.3x10^3±0.3x10^3$	$13.8x10^3±0.9x10^3$ **

Figure 6.5 Effect of Ang II (1.1mg/kg/day for 14 days) compared with vehicle (saline) on TGF-β mRNA expression in WT and αCGRP KO mice. TGF-β mRNA expression in the (a) aorta and (b) heart, kidney, mesenteric resistance vessels and dorsal root ganglia, measured by RT-qPCR, expressed as copies/µl and normalised to SDHA, HPRT-1 and PLA_2. **p<0.01 compared to vehicle treated animals. N=4-5.

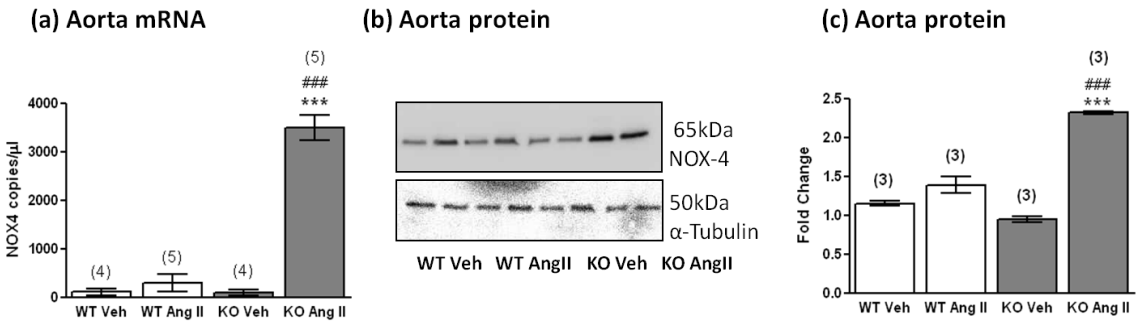
6.7.6 Investigating the effect of Angiotensin II infusion for 14 days on NADPH-oxidase 4 (NOX4) mRNA and protein expression in vascular tissues of WT and αCGRP KO mice.

NOX4 mRNA expression as shown in Figure 6.6 was determined by RT-qPCR in the aorta (figure 6.6a), heart, kidney, MRV and DRG (figure 6.6d) in WT and αCGRP KO mice after 14 days of vehicle or Ang II infusion. Protein NOX4 expression in the aorta (figure 6.6b and c) was determined by Western blotting. With regards to vehicle treated animals, there was no difference in both mRNA and protein expression between WT and αCGRP KO's in all vascular tissues measured. In the WT mice, NOX4 mRNA expression was not significantly upregulated after 14 days Ang II infusion in all tissues analysed (Figure 6.6a and d). This was true also for the hypertensive αCGRP KO mice in the kidney, DRG and MRV (Figure 6.6d). However in the heart, NOX4 mRNA was significantly upregulated in hypertensive αCGRP KO mice in comparison to their vehicle controls ($p<0.001$) and hypertensive WT mice ($p<0.05$, Figure 6.6a). This NOX4 mRNA expression was significantly more elevated in the aortas of hypertensive αCGRP KO mice when compared to both KO vehicle and hypertensive WT mice ($p<0.001$) as shown in Figure 6.6a. Following on from this, NOX4 protein expression in the aorta was determined by Western blotting using the NOX4 antibody donated by Prof. Ajay Shah. Figure 6.6a illustrates the relative amount of NOX4 protein expressed in aortas from WT and αCGRP KO mice treated with either vehicle or Ang II for 14 days. Statistical analysis showed no difference between hypertensive WT mice and their vehicle treated controls. However NOX4 protein was elevated nearly 2.5 fold compared to both αCGRP KO vehicle controls and hypertensive WT mice ($p<0.001$).

Localisation of NOX4 in the thoracic aorta was determined by immunohistochemistry as shown in Figure 6.7. After 14 days with vehicle or Ang II infusion, animals were killed by cervical dislocation and organs collected for post analysis. To observe aortic morphology, the thoracic aorta was excised and washed in saline prior to being fixed in paraformaldehyde and later embedded into paraffin blocks. Sections were then cut and stained for NOX4 by immunohistochemistry as described previously in Chapter 2. When looking at the morphology under the microscope we were able to identify which cell types were positively stained for NOX4 in comparison to the negative control (Figure 6.7a) as

positive staining was brown. We were then able to identify any difference in amount of staining (relative NOX4 expression) between each treatment group; WT vehicle (b), WT Ang II (c), KO Veh (d) and KO Ang II (e). In order to quantify these results, densitometry was used to quantify total positive staining in the whole section measured (Figure 6.7f), and also separate the staining per cell type including endothelial cell (g) smooth muscle cell (h) and surrounding collagen and adventitia (i) as shown in Figure 6.7(g-i). Localisation of NOX4 was shown to be predominantly found in the adventitia and surrounding collagen of the vessel wall, and statistical evaluation showed that NOX4 expression was significantly upregulated in the adventitia of αCGRP KO mice after Ang II infusion when compared to their vehicle counterparts ($p<0.05$).

Based on the findings in figure 6.7, scatter plots were compiled to identify whether there was any relationship between NOX4 and collagen III expression in the aorta at 14 days following Ang II infusion due to NOX4 expression being predominantly found in the collagen and adventitial layer surrounding the vessel wall. The results of these are illustrated in figure 6.8. No direct relationship was found in the vehicle treated groups (figure 6.8 a and c). This was also similar for Ang II infused WTs (figure 6.8b) as although there was an increase in collagen expression, there was no increase in NOX4 expression in these mice. However when we look at the results for the hypertensive αCGRP KO mice, the linear regression and R^2 value of 0.9885 suggests that there is a strong correlation whereby NOX4 expression increases as collagen expression is enhanced following Ang II infusion in the absence of αCGRP.



(d)

Organ	WT Veh	WT Ang II	KO Veh	KO Ang II
Heart	70.2±5.4	96.4±9.9	62.9±5.7	144.2±10.5 *** #
Kidney	$1.2x10^4±0.4x10^4$	$1.0x10^4±0.4x10^4$	$1.2x10^4±0.6x10^4$	$2.2x10^4±0.5x10^4$
MRV	24.8±21.9	34.1±11.7	13.0±0.5	51.9±6.6
DRG	13.2±6.4	6.0±0.8	9.2±6.0	7.0±1.3

Figure 6.6 Effect of Ang II (1.1mg/kg/day for 14 days) compared with vehicle (saline) on NOX4 mRNA expression in WT and αCGRP KO mice. NOX4 mRNA expression in the (a) aorta and protein NOX4, expressed as fold change relative to the vehicle treated controls and normalised to α-tubulin loading control (b and c). NOX4 mRNA expression in the heart, kidney, mesenteric resistance vessels and dorsal root ganglia (d), measured by RT-qPCR, expressed as copies/µl and normalised to SDHA, HPRT-1 and PLA_2. ***p<0.001 compared to vehicle treated animals and #p<0.05, ###p<0.001 compared to WT Ang II treated animals. N=4-5.

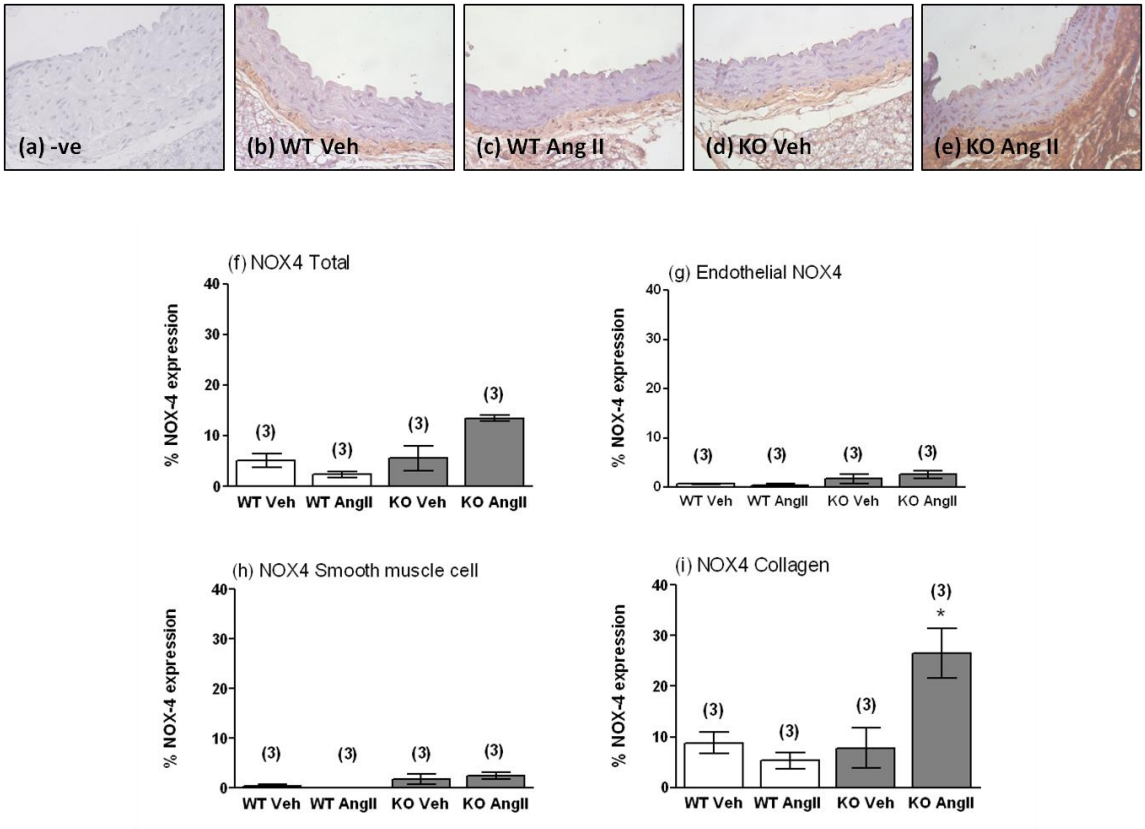


Figure 6.7 Localisation of NOX4 in the aorta of WT and αCGRP KO mice and the effect of Ang II (1.1mg/kg/day for 14 days) compared with vehicle (saline) on NOX4 expression in the aortas of WT and αCGRP KO mice. Immunohistochemical staining of NOX4 in the thoracic aorta wall at 200x magnification. (a) negative control (b) WT vehicle (c) WT Ang II (d) KO Veh (e) KO Ang II and expressed as % positive NOX4 expression of total thoracic section. Quantitative analysis of aortic NOX4 expression in (f) the whole section (g) the endothelial cells (h) smooth muscle cells and (i) surrounding collagen and adventitia. Statistical evaluation of mean ± SEM by ANOVA + Bonferroni's test where *$p<0.05$ compared to vehicle treated animals. N=3.

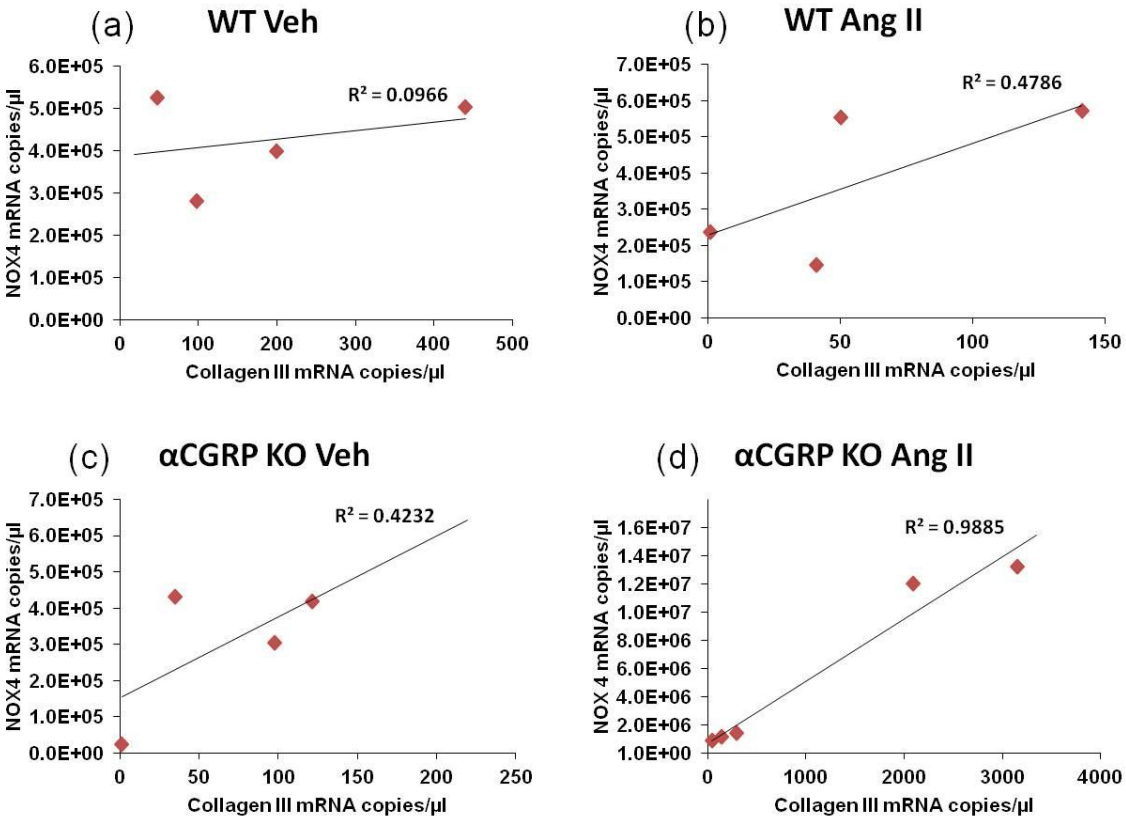


Figure 6.8 Scatter plots showing the correlation between NOX4 and Collagen III mRNA expression in the aortas of WT and αCGRP KO mice following vehicle or Ang II (1.1mg/kg/day for 14 days). Scatter plot (in red) with predicted linear regression as shown in the line of best fit (black) for aortic NOX4 and Collagen III mRNA expression N=4-5.

6.8 Summary

- At baseline (vehicle treatment), there was no difference in levels of oxidative stress markers in αCGRP KO mice when compared to WTs.

- After Ang II infusion, HO1 mRNA expression was increased in aorta of hypertensive mice, this being significantly elevated in αCGRP KOs, however this significance was not observed when protein levels were measured. HO-1 mRNA expression was significantly elevated in the kidneys of hypertensive WT and CGRP KO mice.

- SOD1 mRNA expression was significantly increased in the aortas of hypertensive mice; however there was no difference between WT and αCGRP KOs. SOD1 mRNA was significantly increased in hearts of hypertensive αCGRP KO mice.

- GPX mRNA was elevated in the aorta and hearts of hypertensive animals compared to their matched vehicle controls. When comparing the hypertensive αCGRP KOs to the WTS, the αCGRP KO was more susceptible to a larger increase in GPX mRNA expression in the aorta.

- NOX2 mRNA expression was elevated after Ang II infusion, however this expression is significantly elevated in the aorta and hearts of αCGRP KO mice.

- In the DRG, TGF-β mRNA was elevated in both WT and αCGRP KO mice in the onset of hypertension ($p<0.01$), however this upregulation is similar in both genotypes.

- NOX4 mRNA is upregulated in the aorta and hearts of hypertensive animals, however this expression is significantly more elevated in the αCGRP KO mice compared to WTs. Aortic protein NOX4 levels were also confirmed by protein which showed similar trends. When localised, NOX4 protein expression was predominantly found in the adventitia surrounding the vessel wall of the aorta and scatter plots suggest a relationship between collagen III and NOX4 expression in the absence of αCGRP.

6.9 Discussion

6.9.1 HO1, Ang II and CGRP

The findings from this study have shown that after Ang II infusion, HO1 mRNA expression was increased in aorta of hypertensive WT mice, which was shown previously by Ishizaka *et al*. in 1997 in the rat aorta. However when we observed HO-1 expression in αCGRP KO mice, deletion of αCGRP led to significant upregulation in HO-1 mRNA in the onset of hypertension in KO mice compared to Ang II treated WTs. This significance was lost when protein levels were measured by Western blot, unlike Ishizaka who showed that the HO-1 mRNA was indeed translated into protein in the rat aorta. The difference in our findings to theirs may be due to time point or choice of species. They showed increased protein HO-1 expression after 7 days; however this protein expression may indeed decrease by day 14, therefore time dependent studies would be useful in determining this.

We also found HO-1 mRNA expression to be significantly elevated in the kidneys of hypertensive WT and αCGRP KO mice after Ang II, findings which are similar to that of Kelsen *et al.* in 2008 who showed increased HO-1 expression in the TALH cells from the kidney. The role of the renovascular system and the kidney in the long term regulation of BP has been vastly studied and is well understood (Hosick and Stec, 2011; Guyton, 1996). Given the importance of the kidneys in the regulation of BP, it is feasible to suggest that renal HO-1 may be of

importance in this BP regulation due to its role in regulating intrarenal blood flow under basal conditions and in response to vasoconstrictors (Kaide *et al.* 2004; Zou *et al.* 2000). It has been shown that blockade of renal HO-1 results in the development of salt-sensitive hypertension (Li *et al.* 2007). In addition to this, intrarenal infusion of HO-1 for 2 days prevents the development of Ang II induced hypertension in mice (Vera *et al.* 2007). HO-1 induction has also been shown to prevent the development of renovascular hypertension in rats which had undergone the 2K1C procedure (Botros *et al.* 2005). The development of the HO-1 deficient mouse has also played a pivotal role in identifying the role for HO-1 in models of hypertension. HO-1 KO mice were shown to display exacerbated BP responses in both DOCA-salt and renovascular models of hypertension in comparison to their WT littermates (Nath *et al.* 2007; Wiesel *et al.* 2001).

It is thought that the potential mechanism by which HO-1 lowers BP is thought to be through the production of CO which has positive effects on renal blood flow either directly or indirectly through decreased superoxide production. Studies in cultured TALH cells have shown that increased HO-1 and CO production can inhibit Ang II induced superoxide production (Kelsen *et al.* 2008). Ang II induced superoxide production was also shown to be lowered in mice receiving CO, alongside a decrease in BP, and it is thought that the mechanism by which CO does this may either be via the cyclic cGMP pathway which promote vasodilatation and thus reduces BP or through MAPK signalling to reduce inflammation and target organ injury (Jin *et al.* 2006; Tayem *et al.* 2006; Vera *et al.* 2005).

HO-1 induction has also been shown to prevent the development of Ang II induced hypertension (Yang *et al.* 2004) and reduce BP in the established hypertension induced by Ang II in mice (Vera *et al.* 2007). This reduction in BP has also been shown to be long term as infusion of a HO-1 inducer (hemin) for 3 weeks managed to lower BP and sustain this for the maximum infusion period in SHRs (Wang *et al.* 2006). However the mechanisms by which HO-1 induction can reduce BP in these studies still remains to be addressed.

To conclude, in this study I have shown that in the aorta, deletion of αCGRP causes exacerbated upregulation of HO-1 mRNA but not protein expression in hypertension, perhaps linking a relationship between CGRP and HO-1 at the pre-

transcriptional stage only. It has been shown previously that in rat coronary infarcts treated with monophosphoryl lipid A (MLA, a derivative of endotoxin proposed to promote cardioprotection by release of CGRP), CGRP mRNA expression increased in the heart and DRG in correlation with increasing HO-1 mRNA expression in the same tissue, in addition to elevated plasma CGRP levels also. This CGRP expression was inhibited by ZnPP-9 (zinc protoporphrin IX, a HO-1 inhibitor), suggesting that cardioprotection is mediated by CGRP via the activation of the HO-1 pathway (Peng *et al.* 2001). However in the present study we observe a dramatic increase in HO-1 in response to a lack of αCGRP, perhaps suggesting that in the absence of this potent vasodilator, this antioxidant defence system is exaggerated in order to try and combat against the elevated BP and exacerbated levels of oxidative stress markers.

6.9.2 SOD1, Ang II and CGRP

As previously mentioned, SOD is a powerful antioxidant which catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide. Its role as a major antioxidant has been shown previously where treatment with SOD decreases reactive oxygen species generation and oxidative stress levels in models of bowel disease (Segui *et al*. 2004). In this study I have shown that SOD1 mRNA expression is significantly increased in the aortas of hypertensive mice after 14 days Ang II in comparison to vehicle treated animals; however it should be noted that there was no difference between WT and αCGRP KOs.

In contrast to the aorta, in the heart SOD1 mRNA was significantly increased in hypertensive αCGRP KO mice when compared to Ang II treated mice. These conflicting results may be indicating a relationship between SOD1 and CGRP in some vascular tissues, but not others. However the elevated SOD1 expression in this model indicates a role for this antioxidant in the onset of hypertension which has been previously documented before in this model and also in other hypertension models. In the Ang II model of hypertension, it was shown by Nishiyama *et al.* (2001) that administration of the membrane permeable ROS scavenger and SOD agonist, Tempol, reversed the vasoconstricting actions of Ang II in rats and produced vasodilating actions via an NO-dependent mechanism (Nishiyama *et al.*2001). In another study, 5 days Ang II infusion into rats increased

vascular superoxide production in response to the elevated BP. Treatment with SOD was shown to reduce this elevation of BP by 50mmHg, which in turn also lowered superoxide generation (Laursen *et al.* 1997). Ang II has also been shown to reduce the activity levels and expression of SOD in cardiac fibroblasts (Lijnen *et al.* 2010). In C57BL/6 extracellular SOD (SOD-3) KO mice, Ang II infusion resulted in exacerbated hypertension when compared to respective WT mice, in addition to increased p22phox and NADPH oxidase activity and a reduction in SOD activity, therefore indicating a protective role for SOD in Ang II induced hypertension and oxidative stress (Welch *et al.* 2006).

There is a lack of literature studying the relationship between CGRP and SOD. What is known is that CGRP gene transfer has been shown to suppress ROS generation in the pancreas of diabetic mice by enhancing SOD activity (She *et al.* 2003). Also, in a rat gastric mucosal injury model done in the rat, treatment with capsaite (a TRPV1 activator) attenuated ethanol –induced gastric mucosal injury and cellular apoptosis through an increase of CGRP and SOD activity (Li *et al*. 2012). In this study I have shown that SOD1 expression is indeed increased in the Ang II model of hypertension, thus confirming a role for this antioxidant in combating against oxidative stress in the onset of hypertension. However in the absence of αCGRP, this antioxidant is elevated in the heart suggesting a higher workload for the antioxidant in the absence of this potent vasodilator. This therefore indicates a role for αCGRP in the inhibition of the antioxidants in the onset of hypertension possibly at least in part due to its vasodilatory and positive role in cardioprotection.

6.9.3 GPX, Ang II and CGRP

In this model of hypertension I have shown that GPX mRNA is elevated in hypertensive animals after Ang II infusion; with the αCGRP KO being more susceptible to this increase in mRNA expression in the aorta. A study by Yang *et al.* (2011) showed that vascular adventitial fibroblasts treated with Ang II results in the induction of oxidative stress through the ERK1/2 activation pathway and ROS generation; however there were no reported effects on SOD and GPX levels (Yang *et al.* 2011). Evidence supporting a protective role for GPX in the onset oxidative stress was shown in a study using GPX deficient mice fed an

atherogenic diet. In this model colitis was exacerbated in the KO mice compared to WTs, indicating a protective role for GPX in the onset of oxidative stress (Gao *et al.* 2010). In another study using the same strain of GPX KO mice, when exposed to cigarette smoke these GPX deficient mice displayed enhanced cigarette-smoke induced lung inflammation compared to WT treated mice (Duong *et al.* 2010). In 2009, Zhu *et al.* generated a SOD/GPX double KO strain and reported increased oxidative damage and greater incidence of DNA oxidation and neoplasms in ageing mice lacking SOD and GPX, therefore indicating an important role for these antioxidants in the protection of vascular damage and inflammation (Zhu *et al.* 2009). It has also been shown that relaxation to acetylcholine (Ach) was impaired after 1nmol/L of Ang II in carotid arteries of GPX deficient mice; however this response was prevented in carotid arteries from GPX over expressing mice, suggesting a role for GPX in the Ang II induced effects on vascular function (Chrissobolis *et al.* 2008).

In this study, GPX mRNA has been shown to be elevated in hypertensive animals; with the αCGRP KO being more susceptible to this increase in mRNA expression in the aorta. This result, alongside the results for SOD1 and HO-1, shows that in the onset of oxidative stress induced by Ang II, the antioxidant defence mechanism is significantly enhanced and working actively to counteract the increasing oxidative stress markers such as the NADPH oxidases. However, as oxidative stress progresses, the antioxidant defence system begins to struggle to keep up, therefore modes of drug treatment are essential. In hypertension ACE inhibitors are regularly used in the treatment of essential hypertension to inhibit increasing angiotensin levels and lower BP and studies have shown that they do this through increased SOD and GPX production (De Cavanagh *et al.* 1996). In the absence of αCGRP, the vessel is lacking this potent vasodilatory effect, and therefore the antioxidants have to work even harder to try and counteract and reduce the damage being caused by the exacerbated elevation in BP, therefore suggesting αCGRP to possibly be having a direct protective effect.

6.9.4 NOX2, Ang II and CGRP

In our model of hypertension we have shown that NOX2 mRNA expression is elevated after Ang II infusion, however this expression is significantly elevated in

the aorta and hearts of αCGRP KO mice. NOX2 expression has also been shown previously to be elevated in the aorta of SHRs, salt-induced rat models of hypertension and in mice treated with Ang II (Lassegue and Clempus, 2003; Park *et al.* 2008). In a study by Bendall *et al.* (2008) NOX2 over expressing mice displayed worsened hypertension compared to WT mice after Ang II infusion (Bendall *et al.* 2008). Studies using KO mouse models showed hypertension to be improved when NOX2 was deleted in the 2K1C and DOCA-salt models of hypertension (Jung *et al.* 2004; Fujii *et al.* 2004). However one study using the Ang II induced model of hypertension reported no prevention in the onset of hypertension when using mice deficient in NOX2, although vascular hypertrophy was decreased, suggesting that the effect on BP may occur later on (Dobrien *et al.* 2001). The presence of NOX2 in endothelial cells, fibroblasts and macrophages have also been shown to promote the onset of inflammation and atherogenesis (Siow and Churchman, 2007; Haurani and Pagano, 2007; Csanyi *et al.* 2011). Studies using rabbits fed with high cholesterol atherogenic diets showed a significant elevation in NOX2 expression (Collin *et al*. 2007). In human vessels, NOX2 expression has been shown to be positively correlated with lesion severity in patients with coronary artery disease (Guzik *et al.* 2006), whilst deletion of NOX2 in mouse models reduces the severity of aortic lesions and plaque formation in hyperlipidemic mice (Judkins *et al.* 2010). All of these studies together provide strong evidence for a positive role for NOX2 in the onset of hypertension and atherosclerotic development. In our study, we have shown that NOX2 is elevated in the onset of hypertension, and the lack of the vasodilator αCGRP is increasing the oxidative stress and hence NOX2 production. CGRP has previously been shown to downregulate NADPH oxidase expression and oxidative stress in Ang II-induced endothelial progenitor cells, suggesting a direct protective effect (Zhou *et al.* 2010). This result has also been shown in the ischaemia-reperfused C57BL/6 mouse intestine treated with Ang II inhibitors and CGRP via intravital infusion (Yusof *et al.* 2007). Cultured rat aortic VSMCs treated with Ang II showed markedly elevated ROS production through activated NADPH oxidase. However this increase was attenuated by pre-treatment with $CGRP_{8-37}$ (Liu *et al.* 2006). These results add to the increasing evidence that CGRP is protective in the onset of hypertension, possibly via the activation of the NADPH oxidase pathway which in turn causes the upregulation of the antioxidant defence mechanisms to try and counteract the inflammatory and oxidative response.

6.9.5 TGF-β, Ang II and CGRP

When reviewing the literature discussing the role of TGF-β in the onset of hypertension, there are different views based on different experimental models, however it is generally suggested that TGF-β is involved in the development of hypertension through vascular remodelling and the development of left ventricular hypertrophy. When measuring left ventricle mRNA in rats, TGF-β expression did not differ between SHR (Ohta *et al.* 1994; Brooks *et al.* 1997) and DOCA-salt treated rats when compared to normal rats (Kim *et al.* 1994), however this mRNA expression was elevated in stroke-prone SHRs (Nishikawa, 1998; Kim *et al.* 1996) and post MI rats (Everett *et al.* 1994), which was also shown be inhibited by treatment with ACE inhibitors (Tzanidis *et al.* 2001). With regards to Ang II, this peptide has been shown to elevate TGF-β levels independently of the increased BP and in turn causes extra cellular matrix deposition (Yoo *et al.* 1998). Ang II has also been shown to cause a dose dependent increase in TGF-β mRNA expression in cultured rat aortic VSMCs (Itoh *et al.* 1993). It has also been shown in the SHR that decreasing NO production during the onset of elevated BP causes a marked increase in TGF expression (Wilcox *et al.* 1998). This increase in TGF expression was blunted when SHR were treated with ARBs (Welch *et al.* 2001).

In humans, patients suffering from essential hypertension showed elevated TGF-β mRNA and protein levels (Porreca *et al.* 1997). This overproduction of TGF-β may be due to various factors including elevated BP, overproduction of Ang II or perhaps even shear stress. This elevated TGF-β expression was also reported in the plasma of patients with left ventricle hypertrophy, and again was decreased when treated with ACE inhibitors (Laviades *et al.* 2000; Scaglione *et al.* 2005).

When comparing the literature to this study, we did not observe any differences in TGF-β mRNA in the aorta or heart, however in the DRG, TGF-β mRNA was elevated in both WT and αCGRP KO mice in the onset of hypertension ($p<0.01$), and this upregulation was similar in both genotypes. As there was no difference in TGF-β mRNA expression between WT and αCGRP KO mice, we can assume that αCGRP does not have a relationship with TGF-β in the onset of hypertension, and to date there is no literature to oppose or confirm this conclusion.

6.9.6 NOX4, Ang II and CGRP- Is there a relationship between CGRP and NOX4?

NOX4 differs from the other NOX isoforms in that it only requires interaction with p22phox in order to form a fully functional protein, In addition to this, all NOX isoforms are known to predominantly generate superoxide which in turn gives rise to peroxynitrite formation. However in the case of NOX4, hydrogen peroxide is the predominant product generated (Brandes *et al.* 2010), which activates eNOS. The differences between NOX4 and the other NOX isoforms therefore may be of importance in the onset of vascular pathophysiology and remodelling in response to oxidative stress (Cai *et al.* 2002).

NOX 4 mRNA has been shown previously to be expressed in all cell types of the vascular wall, with this expression being higher than any other NOX homologue (Clempus *et al.* 2007; Haurani *et al.* 2008; Miller *et al.* 2005). In our study we have also found NOX 4 expression in all cell types of the vascular wall (figure 6.7), however this expression is predominantly in the adventitial fibroblasts, with increases in NOX4 expression possibly being linked to the correlating increases in collagen expression and formation in the absence of αCGRP as shown in Figure 6.8d. Studies to identify the signalling pathway of NOX4 have shown that p38MAPK is a downstream target of NOX4, and NOX 4 has also been shown to activate the Ras/ERK pathway. NOX 4 has also been suggested to be involved in Rho activation in the VSMCs (Goettsch *et al.* 2009; Djordjevic *et al.* 2005; Lyle *et al.* 2009). With regards to NOX4 activation, this isoform has been shown to be upregulated and activated by TGF-β, with TGF-β being primarily responsible for NOX 4 activation in response to external stimuli including hypoxia (Brown and Griendling, 2009; Ismail *et al.* 2009), pulmonary hypertension (Fujii *et al.* 2007) and in the vascular healing phase following angioplasty (Xu *et al.* 2008). However in this study we did not observe any increase in TGF-β in tissues displaying elevated NOX4 expression, suggesting that either our time point is too late to detect an increase in TGF-β expression, or NOX4 is not activated via TGF-β in this model of hypertension.

It has been speculated recently as to whether NOX4 is protective or damaging in the onset of vascular diseases due to conflicting data and the need to expand this research in order to draw solid conclusions. As already mentioned at the

beginning of this chapter, the role of NOX4 in hypertension is not well understood due to a limited number of findings, some showing increased NOX4 mRNA expression in hypertensive WTs treated with Ang II (Wingler *et al.*2001; Mollnau *et al* 2002), whilst others reported decreased NOX4 mRNA expression in WT mice (Lassegue *et al.* 2001) or even unchanged NOX protein levels in rats (Wind *et al.* 2010) after Ang II infusion.

In terms of protection, NOX4 has previously been linked to the inhibition of vascular cell growth as growth promoting stimulants such as Ang II, platelet-derived growth factor (PGDF), IL-1β and thrombin have previously been shown to downregulate NOX4 mRNA in VSMCs and adventitial fibroblasts (Haurani *et al.* 2008; Ellmark *et al.* 2005). NOX4 has also been shown to inhibit apoptosis and promote growth of microvascular endothelial cells *in vitro* (Datla *et al.* 2007). Transgenic endothelial-targeted NOX4 over expressing mice have also been used in studies showing NOX4 to be protective and have an enhanced response to acetylcholine-induced vasodilatation and subsequent reduction in BP compared to WT littermate controls (Ray *et al.* 2011). With the use of the NOX4 KO mouse, NOX4 has also been shown to protect against chronic load-induced stress in the heart by angiogenesis (Zhang *et al.* 2010).

In other studies however, NOX4 has been reported to be damaging and enhance cell growth as proliferation in VSMC in response to hypoxia was shown to occur via TGF-β and NOX4 activation (Ismail *et al.* 2009; Djordjevic *et al.* 2005). Ago *et al.* showed that NOX4 was upregulated by Ang II, which in turn caused myocardial dysfunction in mouse cardiac myocytes (Ago *et al.* 2010). Sima *et al.* also showed that oxidized low-density lipoprotein (oxLDL) enhances NOX4 expression in human aortic SMCs (Sima *et al*. 2010).

In this study we have shown upregulation of NOX4 mRNA in the aorta and hearts and increased NOX4 protein in the aorta of hypertensive animals in response to increasing BP induced by Ang II. However this expression is significantly more elevated in the αCGRP KO mice compared to WTs. When observing this result and linking it to the other results previously shown in this study, we can conclude that after Ang II infusion, hypertension occurs with a markedly increase in BP and vascular remodelling of the aorta. Alongside this remodelling there is a shift in the redox state and a marked elevation in oxidative stress in WT animals. However in

the absence of αCGRP, this elevation in BP, vascular remodelling and oxidative stress is significantly more predominant, with the most significant upregulation being NOX4. When we localise the NOX4 in the aorta, it is predominantly found in the proliferating collagen and adventitia of the vessel, and indicates a correlation between collagen formation and NOX4 expression, and possibly a relationship between this and CGRP. However further research is required to better understand the possible relationship between CGRP and NOX4 in Ang II-induced hypertension.

6.10 Conclusion

In this chapter, by using RT-qPCR to measure mRNA gene expression and Western blotting to confirm transfer of mRNA expression into protein, I have shown that the Ang II induced model of hypertension utilised in this study induces oxidative stress by day 14 in response to the elevation of BP and vascular hypertrophy. At baseline (vehicle treatment), there was no difference in levels of oxidative stress markers in αCGRP KO mice when compared to WTs. When focussing specifically on the aorta (the main tissue of interest for this study), HO-1, GPX, SOD1, NOX2 and NOX4 mRNA expression was upregulated in the onset of hypertension in both WT and αCGRP KO mice. However in the absence of αCGRP, hypertensive KO mice displayed exacerbated increases in GPX, NOX2 and NOX4 expression compared to Ang II treated WTs. This data therefore provides evidence for a potentially protective role for αCGRP in the onset of oxidative stress in the Ang II induced model of hypertension, with the possibility of a link between CGRP and NOX4. However the precise mechanism for this is as yet unclear and thus further experiments are required to better the understanding of the findings in this chapter.

CHAPTER SEVEN: THE EFFECTS OF ANGIOTENSIN II INFUSION FOR 28 DAYS ON ARTERIAL BP, VASCULAR HYPERTROPHY AND OTHER CARDIOVASCULAR MARKERS OF HYPERTENSION IN WT AND CGRP KO MICE

7.1 Introduction

The development of Ang II (1.1mg/kg/day) induced hypertension and vascular hypertrophy and inflammation and its effects on both WT and αCGRP KO mice has now been established in this study, as discussed in the previous results chapters. We observed exacerbated hypertension in αCGRP KO mice, alongside increased vascular remodelling, inflammation and oxidative stress, suggesting that αCGRP plays a protective role in the onset of hypertension induced by Ang II. However during the duration of this study it was speculated as to whether this protective role αCGRP was playing was perhaps time-dependent. Structural vascular changes in Ang II infusion has previously been shown to be both dose and time dependent when in rats infused with Ang II for either 4 or 12 weeks. It has been shown that when treated with the same dose of Ang II for 4 or 12 weeks, the vascular changes progress more in 12 weeks, thus causing a more severe hypertensive state. However if using two different doses of Ang II; a high dose (200ng/kg/min) for 4 weeks and a lower dose (50ng/kg/min) for 12 weeks, the end point results in this case are in fact similar (Simon *et al.* 1998). This was also shown in rats in a study looking at time and dose-dependent effects of Ang II on left ventricular cardiomyocytes (Fabris *et al.* 2007). In the dose-response study, Ang II was infused at doses of 100, 200, 400, 800 and 1200 ng/kg per min for 14 days. In the time-dependent study, rats infused with Ang II at doses of 200 and 400 ng/kg per min for 7 and 14 days. Results from this study showed systolic BP and left ventricular mass was increased in a dose-dependent manner in Ang II-infused rats and cardiac apoptosis was influenced by the timing of Ang II infusion (Fabris *et al.* 2007).

It has also been speculated as to whether the protective effects of CGRP are time-dependent in hypertension due to conflicting findings in human plasma levels in hypertension. As previously discussed in Chapter 3, in human studies, there is

debate as to whether circulating plasma CGRP levels increase, decrease or remain unchanged in hypertension. It was previously shown that plasma CGRP levels were decreased in with essential hypertension (Tang *et al.* 1989; Portaluppi *et al.* 1992), however borderline essential hypertensive patients show no significant change in circulating plasma CGRP levels (Lemne *et al.* 1994). On the other hand, Masuda *et al.* showed that increased plasma CGRP levels in hypertensive patients were restricted to a controlled sodium diet compared to normal patients (Masuda *et al.* 1992). In addition to this, IV infusion of Ang-II (sequential doses of 8, 16, and 32 ng/kg.min, each dose for 20 min to normotensive males caused dose-dependent increases of circulating CGRP in addition to elevated BP (Portaluppi *et al.* 1993). In the Ang II model of hypertension, dose-dependent increases in BP and plasma CGRP concentrations occur, suggesting that Ang II and CGRP may not operate independently, but in fact interact with each other either directly or indirectly to regulate the cardiovascular system (Portaluppi *et al.* 1993). We hypothesised that CGRP production may be decreased under conditions of continued Ang II induced hypertension. It is not known what happens to CGRP over a longer period of time, and this study was designed to investigate this. We wanted to identify whether circulating plasma and tissue mRNA expression of CGRP continued to increase in WT mice as hypertension progressed. Or did the opposite happen, whereby the sensory nerve or other sources have tried its best to counteract the increasing BP by releasing increasing amounts of CGRP to cause vasodilatation, but by 28 days CGRP levels are reduced and the hypertension progresses further. If this is the case then the BP of the WT mice would end up catching up with that of the αCGRP KO mouse by day 28 of Ang II infusion.

To refresh on the results found in the 14 day Ang II induced model of hypertension, and the potentially protective role of αCGRP in this model, the main findings are now reviewed.

Chapter 3-Blood Pressure. Age matched WT and αCGRP KO mice did not differ in baseline BP under basal conditions. However, after 14 days Ang II infusion, BP was significantly elevated in both WT and αCGRP KO mice, this hypertension being significantly exacerbated in the hypertensive αCGRP KO mice compared to

hypertensive WT animals. Heart to body weight ratio was significantly increased in hypertensive WT but not αCGRP KO mice.

Chapter 4- CGRP and its receptor. αCGRP mRNA expression was upregulated in the aorta, heart, kidney, MRV and DRG in WT mice after Ang II infusion for 14 days. In contrast, βCGRP mRNA was downregulated in the aorta, but upregulated in the kidney and DRG after Ang II infusion. Circulating total plasma CGRP (α and β) showed an increasing trend after Ang II infusion in both WT and αCGRP KO mice, however this was not significant. CLR mRNA expression was significantly increased in the aortas of both WT and αCGRP KO after Ang II. RAMP1 mRNA expression was not increased in the aorta, but increased in the hearts of WT but not αCGRP KO animals after Ang II. No trends were observed in other tissues, with low copy numbers suggesting low abundance of RAMP1. RAMP2 and RAMP3 mRNA expression did not change between groups.

Chapter 5- Vascular hypertrophy and inflammation. eNOS mRNA expression was downregulated in the aorta in the onset of hypertension with αCGRP KOs showing an increased response to this eNOS downregulation after Ang II infusion. ET-1 mRNA expression was also reduced in the aorta in hypertension. This was significant in WTs but not αCGRP KO mice. However there were no differences in circulating plasma ET-1 concentrations after Ang II infusion.

Aortic VCAM-1 mRNA and protein expression was significantly upregulated in the onset of hypertension, this being significantly higher in hypertensive αCGRP KO mice compared to WTs. MCP-1, ICAM-1, KC (mouse IL-8), IL-10 and TNF-α expression did not differ in WT and αCGRP KO mice in this model. Plasma IL-1 expression was increased in both WT and CGRP KO mice in the onset of hypertension, this significant in the αCGRP KO mice. Plasma IL-12 expression was also increased in both WT and αCGRP KO mice in the onset of hypertension, however there was no difference in IL-12 expression in the absence of αCGRP during hypertension.

IL-6 mRNA expression was significantly increased in aortas of WT but not αCGRP KO mice in the onset of hypertension. Plasma IL-6 expression was raised in the onset of hypertension, however unlike mRNA expression, this elevation was significantly higher in the absence of αCGRP when compared to Ang II treated

WTs. No differences in IL-6 mRNA expression were observed in the heart, MRV and DRG. In the kidney, IL-6 mRNA expression was significantly elevated in hypertensive αCGRP KOs, but not WTs. When comparing the two hypertensive groups, deletion of αCGRP caused significant upregulation of IL-6 mRNA in hearts of αCGRP KOs compared to WTs.

Vascular hypertrophy was apparent in the aortas of WT and αCGRP KO mice after Ang II infusion for 14 days, with αCGRP KO mice displaying exacerbated vascular hypertrophy in comparison to WTs, characterized by increased collagen deposition and mRNA expression.

Chapter 6- Antioxidant defence system and oxidative stress markers. HO1, GPX and NOX2 mRNA expression was increased in aorta of hypertensive mice, this being significantly elevated in αCGRP KOs. SOD1 mRNA expression was also significantly increased in the aortas of hypertensive mice; however this time there was no difference between WT and αCGRP KOs. NOX4 mRNA was upregulated in the aorta and hearts of hypertensive mice; however this expression is significantly more elevated in the αCGRP KO mice compared to WTs. Aortic protein NOX4 showed similar trends. When localised, NOX4 protein expression was predominantly found in the adventitia surrounding the vessel wall of the aorta.

7.2 Summary of the background

In the previous results chapters I have shown that in Ang II induced hypertension, at day 14 hypertension is established in both WT and αCGRP KO mice, characterised by increased BP, vascular hypertrophy and inflammation, remodelling of the aorta and an increased production of oxidative stress. However the hypertension was exacerbated in αCGRP KO mice, with these mice showing a significantly larger incidence of vascular remodelling, inflammation and oxidative stress. These results provide evidence for a protective role for αCGRP in the onset of Ang II induced hypertension, although the mechanisms by which this peptide plays a protective role still remain unclear, and it is speculated as to whether this protective role αCGRP is playing is time dependent.

7.3 Hypothesis

Under baseline conditions, αCGRP does not play a role in the maintenance of BP. However, when infused with Ang II for 14 days, αCGRP KO mice display an exacerbated hypertensive phenotype characterised by significantly elevated BP, increased vascular remodelling and increased oxidative stress. We hypothesis that after 28 days of Ang II infusion, these previous results from 14 days are mirrored, with αCGRP KO mice displaying an increasingly stressed phenotype.

7.4 Aims

- To investigate the role of αCGRP after 28 days Ang II (0.9mg/kg/day) infusion on BP and end point cardiac hypertrophy in WT and αCGRP KO mice by monitoring BP using tail cuff plethysmography.

- To assess the impact of Ang II infusion for 28 days on circulating plasma αCGRP levels and vascular CGRP and CGRP receptor mRNA expression in WT and αCGRP KO mice. Plasma levels will be determined by ELISA, whilst mRNA expression will be measured by RT-qPCR.

- To investigate the vasoactive mediator eNOS in the hypertensive WT and αCGRP KO mouse after Ang II infusion for 28 days by RT-qPCR.

- To investigate inflammatory mediators VCAM-1 and IL-6 in the hypertensive WT and αCGRP KO mouse after Ang II infusion for 28 days by RT-qPCR and determine a role for αCGRP in this model of hypertension.

- To assess the impact of Ang II infusion for 28 days on vascular remodelling and collagen expression and identify a role for αCGRP in this model using

histological techniques to observe the morphology of the aorta in the WT and CGRP KO mouse.

- To assess the impact of Ang II infusion for 28 days on markers of ROS and oxidative stress in both WT and αCGRP KO mice by RT-qPCR.

7.5 Results

7.5.1 Determining the baseline BP values of WT and αCGRP KO mice and investigating the impact of Ang II infusion for 28 days on BP of WT and αCGRP KO mice by measuring BP using tail cuff plethysmography

The basal BP of WT and αCGRP KO mice was already established previously as discussed in Chapter 3 and shown in Figure 3.3, however it deemed good practice to take baseline BP recordings for the mice again before conducting the 28 day Ang II study. Again, using tail cuff plethysmography, the protocol and training regime for the BP recordings were carried out as described in Chapter 2 materials and methods. Figure 7.1 displays the baseline BP measurements for WT and αCGRP KO mice. The values shown are a mean of BP recordings taken over a period of ten days. Figure 7.1a shows that there is no significant difference in systolic pressure between WT and αCGRP KO mice. Figure 7.1b shows there is no difference in diastolic BP between WT and αCGRP KOs, and figure 7.1c shows no difference in MAP when comparing WT and αCGRP KO mice under baseline conditions. This data mimics the baseline data previously shown in chapter 3; again showing that there is no statistical difference in the BP of WT and αCGRP KO mice, suggesting that CGRP does not play a role in the maintenance of BP under basal physiological conditions. Again, a very specific training regime was undertaken with controlled experimental conditions including measurements being obtained at the same time of day as previously recorded in the 14 day study, therefore reproducible measurements were obtained.

After baseline BP readings had been recorded, WT and αCGRP KO mice then underwent the surgical procedure to implant the osmotic minipump as described previously. However this time the dose of Ang II was lowered to 0.9mg/kg/day and infusion time was lengthened to 28 days. The lower dose was used because of worries over whether mice would survive 28 days of the higher dose used previously. BP was then monitored over this 28 day time period as shown in figure 7.2. Over the 28 day time period, WT and αCGRP KO mice that were infused with saline did not display any change in systolic (figure 7.2a), diastolic (b) or MAP (c). Mice infused with Ang II however displayed an increasing elevation of BP over the

28 days and all developed hypertension after approximately 7 days from the day of implantation. When we observe the final BP readings recorded on day 27 (Figure 7.3), WT mice displayed elevated systolic (7.3a), diastolic (b) and MAP (c), these values being markedly hypertensive. However again the αCGRP KO mice displayed exacerbated hypertension, with significantly elevated systolic, diastolic pressure compared to Ang II infused WTs (Figure 7.3, $p<0.05$).

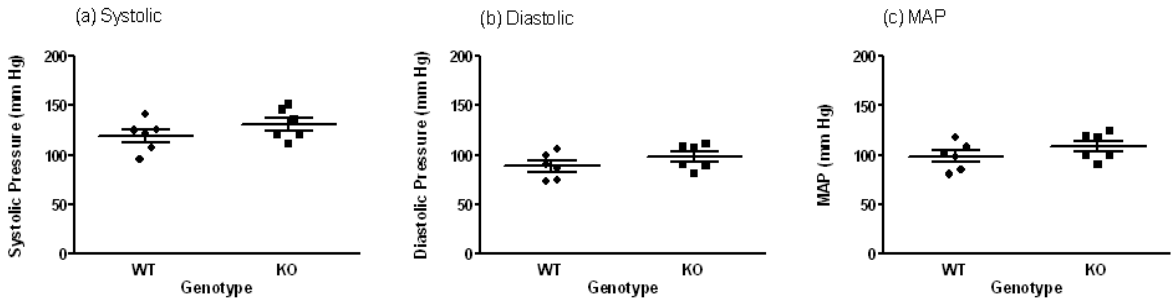


Figure 7.1 Mean arterial pressure and average systolic and diastolic pressure (mmHg) of naive WT and αCGRP KO mice measured by tail cuff plethysmography. Results show (a) mean systolic, (b) mean diastolic and (c) mean arterial pressure (mmHg) in WT and αCGRP KO mice (n=6). Statistical evaluation of mean ± SEM by t-test showed no significant difference.

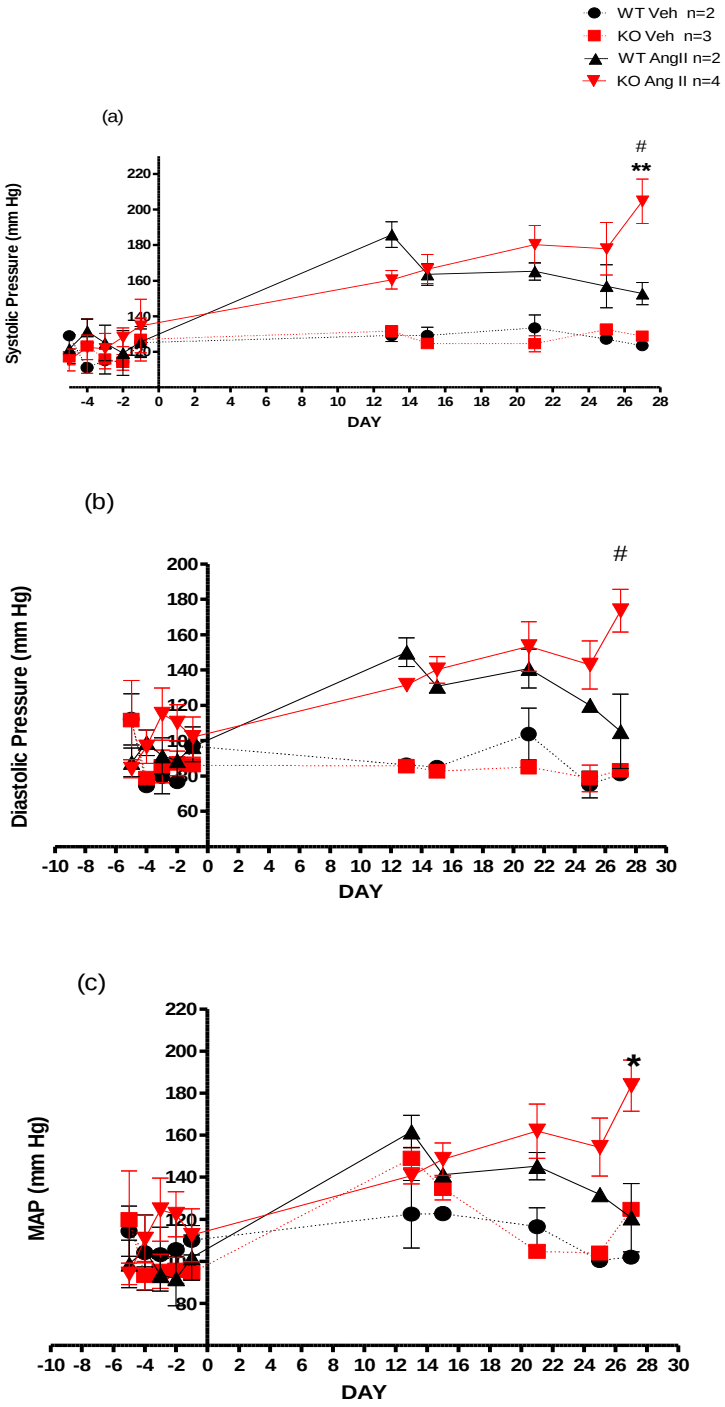


Figure 7.2 Mean arterial pressure (MAP) and average systolic and diastolic pressure (mmHg) of WT and αCGRP KO mice treated with either Vehicle (saline) or Ang II infusion for 28 days, measured by tail cuff plethysmography. (a) Average systolic (b) Average diastolic and (c) Mean arterial pressure (mmHg) in WT and αCGRP KO mice, (n=3-4). Statistical evaluation of mean ± SEM where *p<0.05 and **p<0.1 when compared to vehicle

treated groups and #p<0.05 when compared to WT Ang II treated animals by two-way ANOVA + Bonferroni's test.

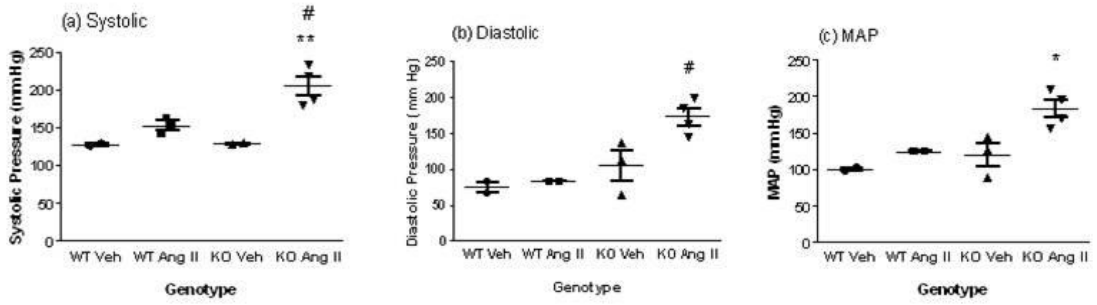


Figure 7.3 Final Mean arterial pressure (MAP) and average systolic and diastolic pressures (mmHg) of WT and αCGRP KO mice treated with either Vehicle (saline) or Ang II infusion for 28 days, measured at day 28 by tail cuff plethysmography. (a) Average systolic (b) Average diastolic and (c) Mean arterial pressure (mmHg) in WT and αCGRP KO mice, (n=3-4). Statistical evaluation of mean ± SEM where *$p<0.05$ and **$p<0.1$ when compared to vehicle treated groups and #$p<0.05$ when compared to WT Ang II treated animals by one-way ANOVA + Bonferroni's test.

7.5.2 Investigating the impact of either vehicle or Ang II (0.9mg/kg/day) infusion for 28 days on end point cardiac hypertrophy in WT and αCGRP KO mice measured by echocardiography

After 28 days infusion of either vehicle or Ang II, mice were anaesthetised and end point markers of cardiac hypertrophy were assessed by echocardiography as described previously in chapter two. Results are shown in Table 7.1. Body weight did not differ between WT and KO mice at any stage of the study. When assessing the different parameters measured, there were no differences between mice infused with vehicle or Ang II, indicating no hypertrophy in the heart in the onset of hypertension in this model. Also there were no cardiac parameter differences between WT and αCGRP KO mice (Table 7.1).

	WT Veh (n=2)	WT Ang II (n=3)	KO Veh (n=2)	KO Ang II (n=4)
Body Weight (g)	24.2±0.3	24.7±1.1	24.0±0.3	23.1±0.4
Stroke volume (µl)	43.0±9.5	35.4±3.7	42.0±1.3	42.3±4.4
Ejection Fraction (%)	61.7±4.0	51.3±2.1	53.7±13.8	54.2±4.8
Cardiac Output (ml/min)	20.7±5.0	18.9±2.5	22.5±0.3	21.0±1.9
LV Mass (mg)	140.8±18.2	146.1±27.9	114.5±28.0	99.9±8.6
Heart:Body weight ratio	5.6±0.3	4.7±0.5	4.8±1.5	4.1±0.3

Table 7.1 Effect of Angiotensin II for 28 days on endpoint markers of hypertension measured by echocardiography in WT and αCGRP KO mice. Developmental markers of 8 week old WT and αCGRP KO mice after 28 days infusion of either vehicle (saline) or Ang II (n=2-4).

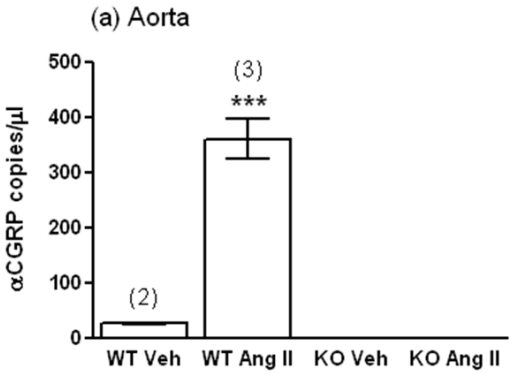
7.5.3 Investigating the effect of Angiotensin II infusion for 28 days on αCGRP and βCGRP mRNA expression in vascular tissues of WT and αCGRP KO mice.

After 28 days infusions with either Angiotensin II (0.9 mg/kg/day) or vehicle (saline), mice were culled by cervical dislocation and organs harvested for analysis. For the purpose of this chapter of the study, aorta, MRV and DRG were carefully excised from the mouse and washed free of blood and immersed in RNAlater before being extracted for RNA and later, reverse transcribed to cDNA (see Chapter 2). RT-qPCR was then carried out to measure RNA expression of αCGRP, βCGRP and the CGRP receptor components in each tissue.

Figure 7.4 shows αCGRP mRNA expression in (a) the aorta and (b) MRV and DRG. All results are expressed as copies/µl of cDNA. Again as expected, no expression was found in αCGRP KO mice, thus again confirming that the mice were indeed absent of αCGRP. αCGRP mRNA expression was significantly upregulated in all tissues of hypertensive WT mice after 28 days Ang II treatment, shown in figure 7.4 ($p<0.001$). As previously observed in chapter 6, copy numbers of αCGRP in the DRG are relatively low, indicating low expression in this tissue, in comparison to the aorta which is displaying higher expression of αCGRP when observing copies/µl (figure 7.4a). However there is significantly more abundance of αCGRP in the mesenteric resistance vessels compared to the aorta, and this expression is increased approximately 300 fold in hypertensive animals in comparison to their matched vehicle treated controls (Figure 7.4b).

Figure 7.5 shows βCGRP mRNA expression in the (a) aorta and (b) MRV and DRG. Again all results are expressed as copies/µl of cDNA. As before, we did expect to observe expression in the αCGRP KO mice as the beta isoforms has not been knocked out in this model. In the aorta (figure 7.5a), after 28 days βCGRP is now significantly upregulated in the hypertensive WT animals ($p<0.001$). However this expression is lost in αCGRP KO animals infused with Ang II when compared to their WT counterparts ($p<0.001$). Similar results are also observed in the DRG (Figure 7.5b). In the mesenteric resistance vessels, βCGRP appears to be upregulated in both hypertensive WT and αCGRP KOs, although this elevation is not statistically significant (Figure 7.5c). It is also worth noting here that the abundance of βCGRP expression measured in the aorta is

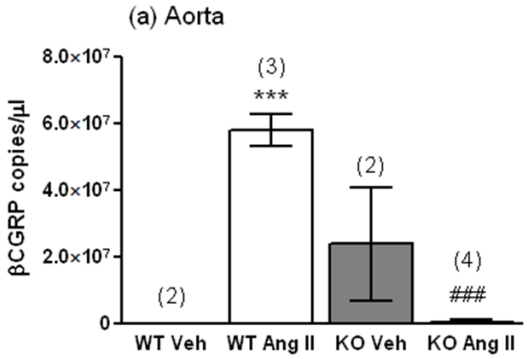
significantly greater than that of αCGRP which is unexpected due to original suggestions that the α isoform is the most predominant in the vasculature, and not the β isoform, which was originally thought to only be predominant in the gut and enteric nerves.



(b)

Organ	WT Veh	WT Ang II	KO Veh	KO Ang II
MRV	2.9±8.0	860±7.5***	0.0±0.0	0.0±0.0
DRG	19±0.0	46.3±5.7 ***	0.0±0.0	0.0±0.0

Figure 7.4 Effect of Ang II (0.9mg/kg/day for 28 days) compared with vehicle (saline) on αCGRP mRNA expression in WT and αCGRP KO mice. αCGRP mRNA expression in the (a) aorta and (b) mesenteric resistance vessels and dorsal root ganglia, measured by RT-qPCR, expressed as copies/µl and normalised to SDHA, HPRT-1 and PLA_2. ***p<0.001 compared to vehicle treated animals. N=2-4.



(b)

Organ	WT Veh	WT Ang II	KO Veh	KO Ang II
MRV	$8.4x10^6 \pm 2.8x10^6$	$14x10^6 \pm 12x10^6$	$1.8x10^6 \pm 1.5x10^6$	$14x10^6 \pm 3.8x10^6$
DRG	$0.01x10^{10} \pm 0.01x10^{10}$	$3.9x10^{10} \pm 2.6x10^{10}$	$0.1x10^{10} \pm 0.1x10^{10}$	$0.001x10^{10} \pm 0.003x10^{10}$ ** ###

Figure 7.5 Effect of Ang II (0.9mg/kg/day for 28 days) compared with vehicle (saline) on βCGRP mRNA expression in WT and αCGRP KO mice. βCGRP mRNA expression in the (a) aorta and (b) mesenteric resistance vessels and dorsal root ganglia, measured by RT-qPCR, expressed as copies/µl and normalised to SDHA, HPRT-1 and PLA_2. **p<0.01 and ***p<0.001 compared to vehicle treated animals, and ##p<0.01 and ###p<0.001 compared to Ang II treated WTs. N=2-4.

7.5.4 Investigating the effect of Angiotensin II infusion for 28 days on circulating plasma CGRP levels in WT and αCGRP KO mice.

At day 28 of the study, and prior to cervical dislocation, mice were anaesthetised with 4% isoflurane (O_2 carrier gas, 4L/min flow rate) and a blood sample (1ml) was collected via cardiac puncture using a 25G needle from the left ventricle. This blood was then spun down for 10 minutes at 2500G, temperature controlled at 4ºC, and plasma was collected and snap frozen in liquid nitrogen. Plasma samples from each animal were extracted and purified before being assayed for αCGRP detection by ELISA and normalised to relative plasma protein concentration, as previously described. Results are shown in Figure 7.6 and expressed as ng/mg of total plasma protein. This time the assay was obtained from Bachem (Rabbit anti-α-CGRP, Cat. No. T4032), and raised against αCGRP only, unlike the assay used in Chapter 6 which detected both α and β CGRP isoforms. However it is worth noting that cross-reactive data obtained by radioimmunoassay applications detected 16.8% cross-reactivity with the βCGRP isoform.

Figure 7.6 illustrates the resulting plasma αCGRP levels in WT and αCGRP KO mice after either vehicle or Ang II infusion for 28 days. After vehicle treatment, which we again can also consider as a baseline value, circulating αCGRP levels were not present in KO mice. However circulating CGRP levels were increased in Ang II induced hypertension after 14 days in WTs, although statistical evaluation of the data does not show any significant difference

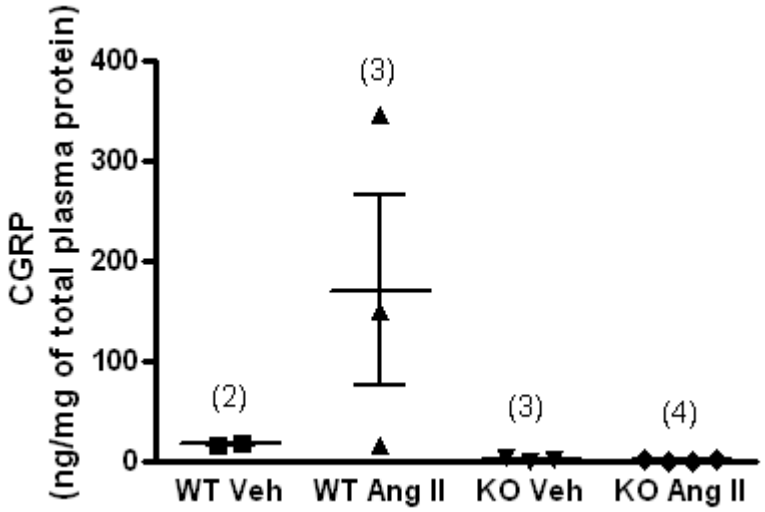
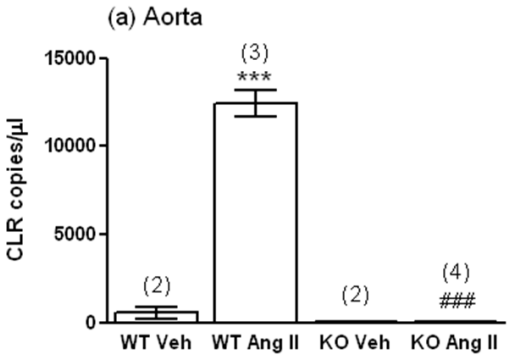


Figure 7.6 Total plasma αCGRP. Total plasma αCGRP concentrations (ng/mg of total plasma protein) in WT and αCGRP KO mice treated with either vehicle (saline) or Ang II (0.9mg/kg/day) for 28 days, measured by an enzyme-linked immunosorbent assay (Peninsula Labs). Statistical evaluation of mean ± SEM by ANOVA + Bonferroni's test showed no significant differences.

7.5.5 Investigating the effect of Angiotensin II infusion for 28 days on the CGRP receptor components mRNA expression in vascular tissues of WT and αCGRP KO mice.

Figure 7.7 shows CLR mRNA expression in the aorta (a) MRV and DRG (b) in WT and αCGRP KO mice treated with either saline or Ang II for 28 days. All results are expressed as copies/µl of cDNA. In the aorta, CLR expression significantly increased in the hypertensive WT mice compared to their vehicle treated counterparts (Figure 7.7a, $p<0.001$). However, unlike the 14 day study where this increase was also observed in hypertensive αCGRP KOs, very little CLR expression was not observed in αCGRP KO mice in this model compared to WT mice treated with Ang II ($p<0.001$). CLR mRNA expression was slightly elevated in the DRG and MRV of KO animals after Ang II treatment, but not significantly so. However this expression was not shown to be elevated in the MRV and DRG of hypertensive WTs (Figure 7.7b).

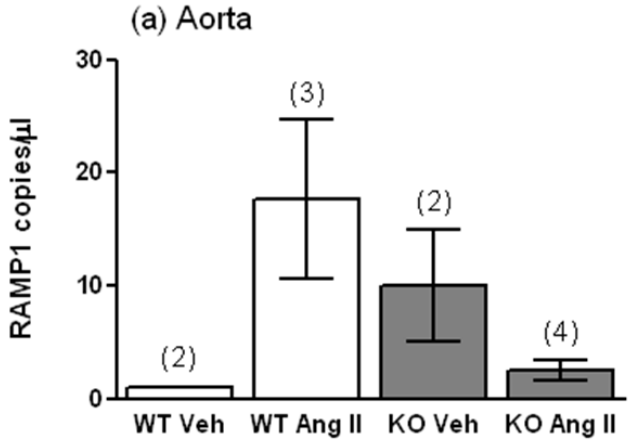
Figure 7.8 shows RAMP1 mRNA expression in the aorta (a), MRV and DRG (b) in WT and αCGRP KO mice after either vehicle or Ang II infusion for 28 days. Although not statistically significant, RAMP1 mRNA was elevated in the aorta of WTs after Ang II infusion, but downregulated in αCGRP KOs (Figure 7.8a). In the MRV and DRG, there were trends showing downregulation of RAMP1 in hypertensive WT and αCGRP KO mice (Figure 7.8b). Again, copy numbers were low in all tissues measured, suggesting low RAMP1 expression in these tissues.



(b)

Organ	WT Veh	WT Ang II	KO Veh	KO Ang II
MRV	$2.2x10^3 \pm 0.5x10^3$	$2.0x10^3 \pm 0.4x10^3$	$0.9x10^3 \pm 0.5x10^3$	$3.4x10^3 \pm 0.7x10^3$
DRG	$1.8x10^3 \pm 0.4x10^3$	$1.1x10^3 \pm 0.2x10^3$	$2.1x10^3 \pm 0.2x10^3$	$3.0x10^3 \pm 0.5x10^3$

Figure 7.7 Effect of Ang II (0.9mg/kg/day for 28 days) compared with vehicle (saline) on calcitonin-like receptor (CLR) mRNA expression in WT and αCGRP KO mice. CLR mRNA expression in the (a) aorta and (b) mesenteric resistance vessels and dorsal root ganglia, measured by RT-qPCR, expressed as copies/µl and normalised to SDHA, HPRT-1 and PLA_2. ***p<0.001 compared to vehicle treated animals, and ###p<0.001 compared to Ang II treated WTs. N=2-4.



(b)

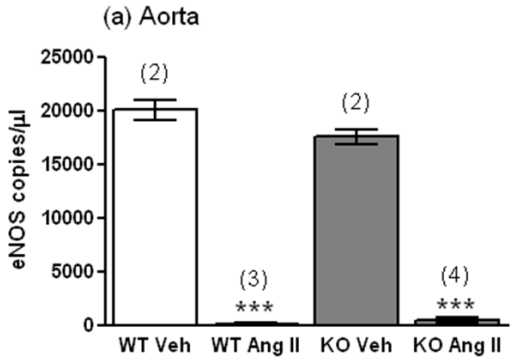
Organ	WT Veh	WT Ang II	KO Veh	KO Ang II
MRV	72±56	53±6.8	104.5±14	67±37.2
DRG	5.5±4.5	3.3±0.8	26.5±13.5	9.2±3.0

Figure 7.8 Effect of Ang II (0.9mg/kg/day for 28 days) compared with vehicle (saline) on RAMP1 mRNA expression in WT and αCGRP KO mice. RAMP1 mRNA expression in the (a) aorta and (b) mesenteric resistance vessels and dorsal root ganglia, measured by RT-qPCR, expressed as copies/µl and normalised to SDHA, HPRT-1 and PLA_2. Statistical evaluation of mean ± SEM by ANOVA + Bonferroni's test showed no statistical difference. N=2-4.

7.5.6 Investigating the effect of Angiotensin II infusion for 28 days on the vasoactive mediators (the NO synthase enzyme, eNOS and ET-1) mRNA expression in vascular tissues of WT and αCGRP KO mice.

Figure 7.9 shows eNOS mRNA expression in the aorta (a) MRV and DRG (b) in WT and αCGRP KO mice treated with either saline or Ang II for 28 days. All results are expressed as copies/µl of cDNA. In the aorta (Figure 7.9a) eNOS expression does not differ between vehicle infused WT and αCGRP KO mice. In the onset of hypertension, eNOS mRNA expression is dramatically diminished in both WT and αCGRP KO mice after 28 days Ang II infusion ($p<0.001$). There is no difference in the very low eNOS level between the hypertensive mice (Figure 7.9a). With regards to the mesenteric resistance vessels and dorsal root ganglia, no differences were observed between the four treatment groups.

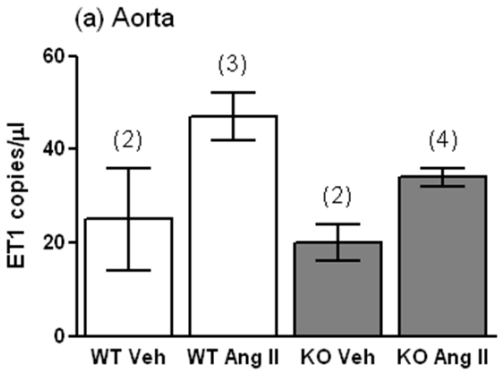
Figure 7.10 shows ET-1 mRNA expression in the aorta (a) MRV and DRG (b) in WT and αCGRP KO mice treated with either saline or Ang II for 28 days. All results are expressed as copies/µl of cDNA. In the aorta (Figure 7.10a), although not significant, there is a trend toward an upregulation of ET-1 mRNA expression in both WT and αCGRP KO mice in the onset of hypertension after Ang II infusion for 28 days, although copy numbers are low. No differences in ET-1 mRNA expression were observed in the mesenteric resistance vessels and dorsal root ganglia from WT and αCGRP KO mice treated with either vehicle or Ang II for 28 days.



(b)

Organ	WT Veh	WT Ang II	KO Veh	KO Ang II
MRV	$28.2x10^3 \pm 0.3x10^3$	$17.3x10^3 \pm 8.9x10^3$	$22.7x10^3 \pm 10.0x10^3$	$25.5x10^3 \pm 8.2x10^3$
DRG	$2.4x10^3 \pm 0.1x10^3$	$2.9x10^3 \pm 0.2x10^3$	$6.4x10^3 \pm 1.7x10^3$	$4.5x10^3 \pm 0.7x10^3$

Figure 7.9 Effect of Ang II (0.9mg/kg/day for 28 days) compared with vehicle (saline) on eNOS mRNA expression in WT and αCGRP KO mice. eNOS mRNA expression in the (a) aorta and (b) mesenteric resistance vessels and dorsal root ganglia, measured by RT-qPCR, expressed as copies/µl and normalised to SDHA, HPRT-1 and PLA_2. ***p<0.001 compared to vehicle treated animals. N=2-4.



(b)

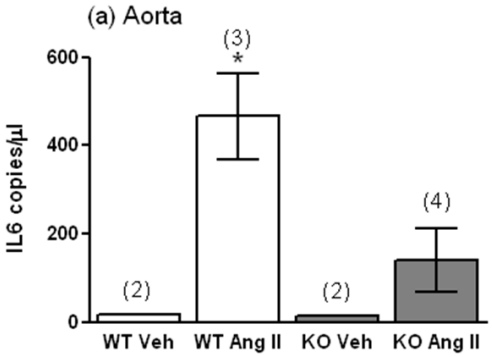
Organ	WT Veh	WT Ang II	KO Veh	KO Ang II
MRV	31.0±5.0	27.0±10.6	37.0±2.0	45.0±8.3
DRG	42.0±1.0	39.3±0.6	45.0±1.0	37.25±4.4

Figure 7.10 Effect of Ang II (0.9mg/kg/day for 28 days) compared with vehicle (saline) on ET-1 mRNA expression in WT and αCGRP KO mice. ET-1 mRNA expression in the (a) aorta and (b) mesenteric resistance vessels and dorsal root ganglia, measured by RT-qPCR, expressed as copies/µl and normalised to SDHA, HPRT-1 and PLA_2. Statistical evaluation by ANOVA + Bonferroni's post test showed no difference between each treatment group. N=2-4.

7.5.7 Investigating the effect of Angiotensin II infusion for 28 days on markers of vascular inflammation (IL-6 and VCAM-1) mRNA expression in vascular tissues of WT and αCGRP KO mice.

Figure 7.11 illustrates IL-6 mRNA expression in the aorta (a) MRV and DRG (b) in WT and αCGRP KO mice treated with either saline or Ang II for 28 days. All results are expressed as copies/µl of cDNA. In the aorta (Figure 7.11a) IL-6 mRNA expression did not differ between WT and αCGRP KO mice treated with vehicle (saline). In Ang II induced hypertension, at day 28 IL-6 mRNA expression was significantly upregulated in WT ($p<0.05$) but not αCGRP KO mice. No differences in IL-6 mRNA expression was observed in the mesenteric resistance vessels and dorsal root ganglia from WT and αCGRP KO mice treated with either vehicle or Ang II for 28 days (Figure 7.11b).

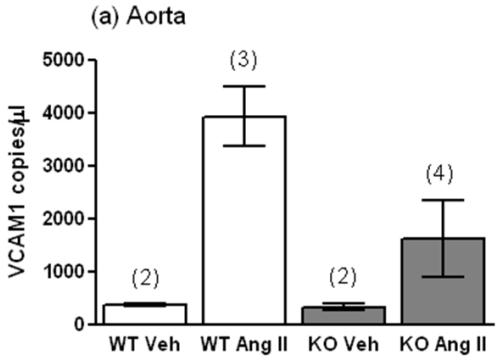
Figure 7.12 shows VCAM-1 mRNA expression in the aorta (a) MRV and DRG (b) in WT and αCGRP KO mice treated with either saline or Ang II for 28 days. All results are expressed as copies/µl of cDNA. In the aorta (Figure 7.12a), VCAM-1 mRNA expression did not differ between vehicle treated WT and αCGRP KO mice. In hypertension induced by Ang II, although not statistically significant, there is a trend showing an increase in VCAM-1 expression. However this upregulation seems to be more prominent in WT mice compared to αCGRP KO mice. No differences in VCAM-1 mRNA expression were observed in the mesenteric resistance vessels and dorsal root ganglia of WT and αCGRP KO vehicle treated mice, or in the onset of hypertension (Figure 7.12b).



(b)

Organ	WT Veh	WT Ang II	KO Veh	KO Ang II
MRV	109.5±3.5	294.7±102.8	224.0±77.0	385.3±127.8
DRG	306.0±19.0	244.3±17.3	260.0±26.0	442.8±90.1

Figure 7.11 Effect of Ang II (0.9mg/kg/day for 28 days) compared with vehicle (saline) on IL-6 mRNA expression in WT and αCGRP KO mice. IL-6 mRNA expression in the (a) aorta and (b) mesenteric resistance vessels and dorsal root ganglia, measured by RT-qPCR, expressed as copies/µl and normalised to SDHA, HPRT-1 and PLA_2. *$p<0.05$ compared to vehicle treated animals. N=2-4.



(b)

Organ	WT Veh	WT Ang II	KO Veh	KO Ang II
MRV	$3.1x10^3±0.2x10^3$	$1.1x10^3±0.5x10^3$	$0.9x10^3±0.9x10^3$	$9.3x10^3±5.0x10^3$
DRG	$2.2x10^3±0.6x10^3$	$1.4x10^3±0.02x10^3$	$2.1x10^3±1.8x10^3$	$1.4x10^3±0.4x10^3$

Figure 7.12 Effect of Ang II (0.9mg/kg/day for 28 days) compared with vehicle (saline) on VCAM-1 mRNA expression in WT and αCGRP KO mice. VCAM-1 mRNA expression in the (a) aorta and (b) mesenteric resistance vessels and dorsal root ganglia, measured by RT-qPCR, expressed as copies/µl and normalised to SDHA, HPRT-1 and PLA_2. Statistical evaluation showed no difference between each treatment group. N=2-4.

7.5.8 Investigating the effect of Angiotensin II infusion for 28 days on vascular hypertrophy of the aorta and aortic collagen III mRNA expression in WT and αCGRP KO mice.

Sections of thoracic aorta were collected from animals on day 28 and fixed and prepared in paraffin blocks before being sectioned and fixed onto polylysine slides for staining as described previously in chapter two. After staining with Masson's Trichrome stain, cross sections of aorta were examined and measured under a microscope to determine whether or not the hypertensive mice in this study exhibited symptoms of vascular remodelling shown in figure 7.13 and if there was any difference between WT and αCGRP KO mice. Analysis of mean aortic width measurements (μm) from Masson's Trichrome stained aortic sections in figure 7.14(a) showed mean aortic wall width to be increased in WTs but significantly increased in αCGRP KO mice infused with Ang II compared to their matched vehicle controls ($p<0.05$). When analysing Collagen 7.14(b) and SMC 7.14(c) width individually, figure 7.14(b) showed no difference in mean collagen width (μm) in WT and αCGRP KO mice infused with Ang II compared to the Vehicle treated groups, however there is a slight trend showing increased collagen in hypertensive αCGRP KOs. Figure 7.14c illustrates the differences in mean SMC width (μm) whereby SMC width was increased in hypertensive animals after Ang II for 28 days, this being significantly elevated in αCGRP KO animals compared to their matched vehicle controls ($p<0.01$).

Figure 7.15 illustrates Collagen III mRNA expression in the aortas of WT and αCGRP KO mice treated with either saline or Ang II for 28 days. All results are expressed as copies/μl of cDNA. In the aorta collagen III mRNA expression did not differ between vehicle treated WT and αCGRP KO mice. In hypertension induced by Ang II, collagen III expression was significantly upregulated in WT ($p<0.05$) and αCGRP KO ($p<0.001$) mice at day 28. When we compare the two hypertensive groups we found that in the absence of αCGRP, mice are more susceptible to increasing collagen III expression in the aorta after 28 days Ang II infusion in comparison to hypertensive WT mice ($p<0.01$).

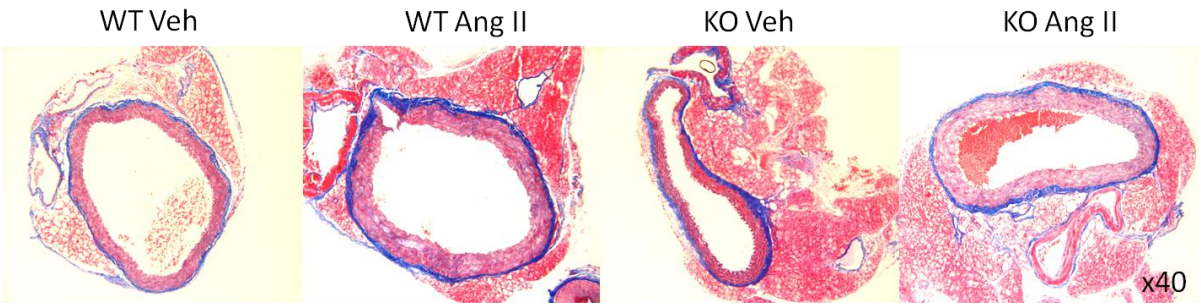


Figure 7.13. The effect of either vehicle or Angiotensin II infusion for 28 days on the aortic wall remodelling of WT and αCGRP KO mice. Showing representative images of aortic wall stained with Masson's trichrome at 40x magnification.

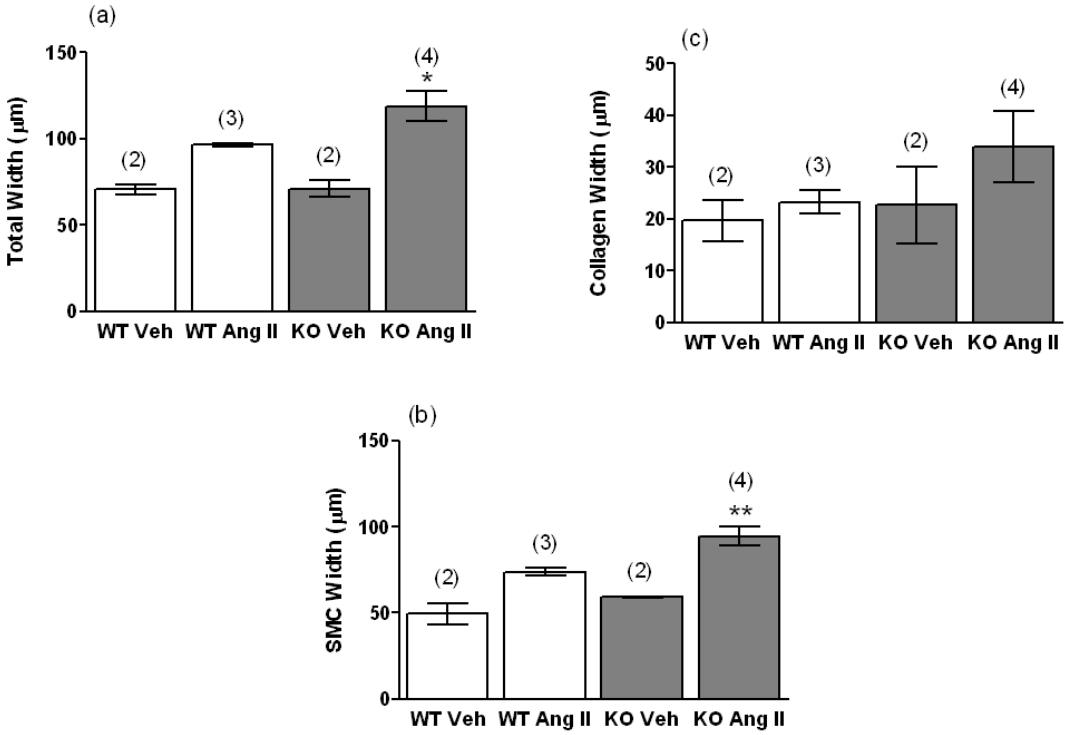


Figure 7.14 Total, SMC and collagen width (µm), measured from aortic sections taken from mixed gender WT and αCGRP KO mice treated with either Vehicle (saline) or Ang II infusion for 28 days. (a) Total width (SMC + collagen) in WT and αCGRP KO mice, (n=2-4). (b) Total SMC width (µm) in WT and αCGRP KO mice, (n=2-4) and (c) Total collagen width (µm) in WT and αCGRP KO mice, (n=2-4). Statistical evaluation of mean ± SEM by 2 way ANOVA and Bonferroni's test, where *=$p<0.05$ and **=$p<0.01$ compared to vehicle treated groups.

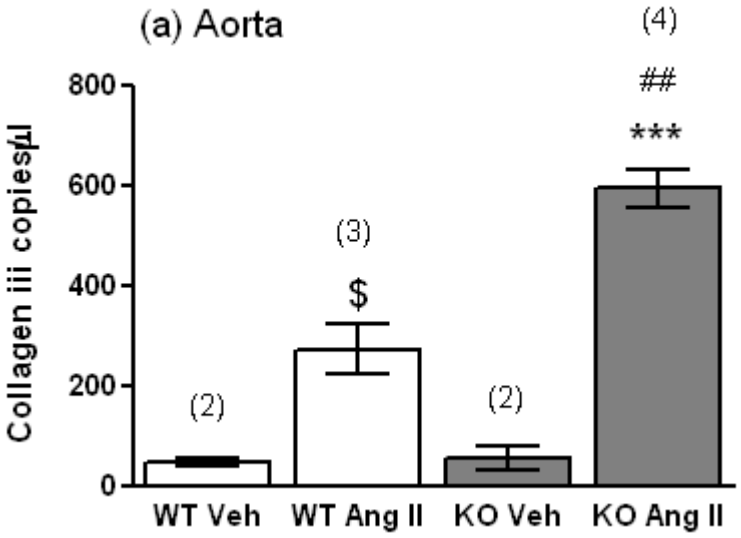


Figure 7.15 Effect of Ang II (0.9mg/kg/day for 28 days) compared with vehicle (saline) on collagen III mRNA expression in WT and αCGRP KO mice. Collagen III mRNA expression in the (a) aorta and (b) mesenteric resistance vessels and dorsal root ganglia, measured by RT-qPCR, expressed as copies/µl and normalised to SDHA, HPRT-1 and PLA_2. $p<0.5 and ***p<0.001 compared to vehicle treated animals, and ###p<0.001 compared to Ang II treated WTs. N=2-4.

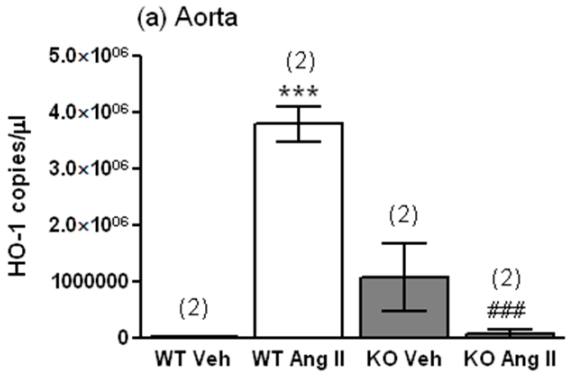
7.5.9 Investigating the effect of Angiotensin II infusion for 28 days on markers of the vascular antioxidant defence system (HO-1, SOD1 and GPX) in vascular tissues by RT-qPCR in WT and αCGRP KO mice.

Figure 7.16 shows HO-1mRNA expression in the aorta (a) MRV and DRG (b) in WT and αCGRP KO mice treated with either saline or Ang II for 28 days. All results are expressed as copies/µl of cDNA. In the aorta (Figure 7.16a), when comparing WT and αCGRP KO vehicle treated mice no differences in expression were observed. In the onset of hypertension, HO-1 mRNA expression was significantly upregulated in Ang II treated WTs ($p<0.001$), however this upregulation was not observed in hypertensive αCGRP KO mice, therefore HO-1 expression was significantly lower in hypertensive αCGRP KO mice compared to hypertensive WTs ($p<0.001$). No differences in HO-1 mRNA expression were observed in the mesenteric resistance vessels and dorsal root ganglia of WT and αCGRP KO vehicle treated mice, or in the onset of hypertension (Figure 7.16b).

Figure 7.17 illustrates SOD1 mRNA expression in the aorta (a) MRV and DRG (b) in WT and αCGRP KO mice treated with either saline or Ang II for 28 days. All results are expressed as copies/µl of cDNA. In the aorta (Figure 7.17a), no differences in mRNA expression were observed when comparing vehicle treated WT and αCGRP KO mice. In the onset of hypertension, SOD-1 mRNA expression was upregulated in WT mice (not significant) and significantly elevated in Ang II treated αCGRP KO mice ($p<0.05$). When comparing the two hypertensive groups, there was no significant difference in SOD1 mRNA expression. No differences in SOD1 mRNA expression were observed in the mesenteric resistance vessels and dorsal root ganglia of WT and αCGRP KO vehicle treated mice, or in the onset of hypertension (Figure 7.17b).

Figure 7.18 displays GPX mRNA expression in the aorta (a) MRV and DRG (b) in WT and αCGRP KO mice treated with either saline or Ang II for 28 days. All results are expressed as copies/µl of cDNA. In the aorta (Figure 7.18a) when comparing WT and αCGRP KO vehicle treated mice, no differences in GPX mRNA expression were observed. GPX mRNA expression was significantly elevated in WT mice after Ang II infusion when compared to their vehicle treated controls ($p<0.01$). However this increase in GPX mRNA expression was not

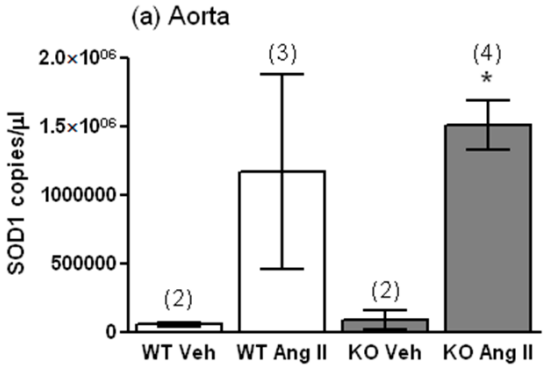
observed in αCGRP KO mice in hypertension at day 28. Instead, this expression was significantly downregulated when compared to the vehicle treated control ($p<0.01$). When comparing the two hypertensive groups together, GPX mRNA expression was significantly elevated in hypertensive WT animals in comparison to αCGRP KOs after 28 days Ang II infusion ($p<0.001$). No differences in GPX mRNA expression were observed in the mesenteric resistance vessels and dorsal root ganglia of WT and αCGRP KO vehicle treated mice, or in the onset of hypertension (Figure 7.18b).



(b)

Organ	WT Veh	WT Ang II	KO Veh	KO Ang II
MRV	$2.0x10^6 \pm 0.3x10^6$	$1.4x10^6 \pm 0.9x10^6$	$1.8x10^6 \pm 0.2x10^6$	$4.3x10^6 \pm 1.1x10^6$
DRG	$2.5x10^6 \pm 0.6x10^6$	$2.0x10^6 \pm 0.5x10^6$	$4.2x10^6 \pm 0.8x10^6$	$3.2x10^6 \pm 1.6x10^6$

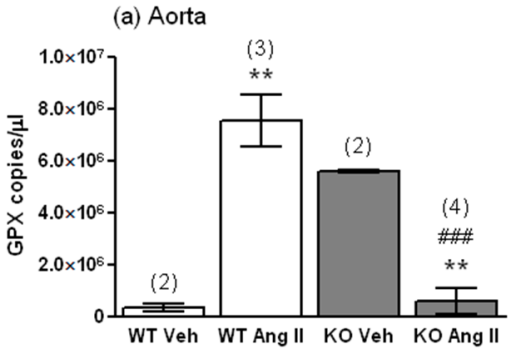
Figure 7.16 Effect of Ang II (0.9mg/kg/day for 28 days) compared with vehicle (saline) on HO-1 mRNA expression in WT and αCGRP KO mice. HO-1 mRNA expression in the (a) aorta and (b) mesenteric resistance vessels and dorsal root ganglia, measured by RT-qPCR, expressed as copies/µl and normalised to SDHA, HPRT-1 and PLA_2. ***p<0.001 compared to vehicle treated animals. N=2-4.



(b)

Organ	WT Veh	WT Ang II	KO Veh	KO Ang II
MRV	$314x10^3 \pm 6.3x10^3$	$353x10^3 \pm 165x10^3$	$378x10^3 \pm 24x10^3$	$350x10^3 \pm 31x10^3$
DRG	$13.9x10^4 \pm 0.9x10^4$	$17.6x10^4 \pm 0.7x10^4$	$23.3x10^4 \pm 1.8x10^4$	$23.3x10^4 \pm 2.9x10^4$

Figure 7.17 Effect of Ang II (0.9mg/kg/day for 28 days) compared with vehicle (saline) on SOD1 mRNA expression in WT and αCGRP KO mice. SOD-1 mRNA expression in the (a) aorta and (b) mesenteric resistance vessels and dorsal root ganglia, measured by RT-qPCR, expressed as copies/µl and normalised to SDHA, HPRT-1 and PLA_2. *p<0.5 compared to vehicle treated animals. N=2-4.



(b)

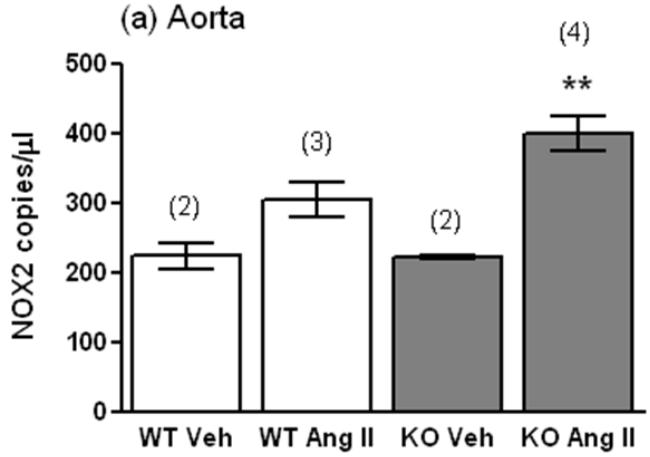
Organ	WT Veh	WT Ang II	KO Veh	KO Ang II
MRV	$6x10^6 \pm 0.1x10^6$	$4.8x10^6 \pm 2.1x10^6$	$4.6x10^6 \pm 1.1x10^6$	$5.6x10^6 \pm 0.5x10^6$
DRG	$3.5x10^6 \pm 0.5x10^6$	$2.0x10^6 \pm 0.1x10^6$	$1.0x10^6 \pm 0.1x10^6$	$2.8x10^6 \pm 0.7x10^6$

Figure 7.18 Effect of Ang II (0.9mg/kg/day for 28 days) compared with vehicle (saline) on GPX mRNA expression in WT and αCGRP KO mice. GPX mRNA expression in the (a) aorta and (b) mesenteric resistance vessels and dorsal root ganglia, measured by RT-qPCR, expressed as copies/µl and normalised to SDHA, HPRT-1 and PLA_2. **p<0.01 compared to vehicle treated animals and ###p<0.001 compared to WT Ang II treated animals. N=2-4.

7.5.10 Investigating the effect of Angiotensin II infusion for 28 days on markers of oxidative stresses (NOX2 and NOX4) in vascular tissues by RT-qPCR in WT and αCGRP KO mice.

Figure 7.19 illustrates NOX2 mRNA expression in the (a) aorta and (b) MRV and DRG in WT and αCGRP KO mice treated with either saline or Ang II for 28 days. All results are expressed as copies/µl of cDNA. In the aorta (Figure 7.19a), when comparing WT and αCGRP KO vehicle treated mice no differences in expression were observed. NOX2 mRNA expression was upregulated in the onset of hypertension, this expression being significantly so in αCGRP KO mice when compared to their vehicle treated controls ($p<0.05$). However when comparing the two hypertensive groups, no significant differences in NOX2 expression after Ang II infusion were observed. No differences in NOX2 mRNA expression were observed in the mesenteric resistance vessels and dorsal root ganglia of WT and αCGRP KO vehicle treated mice, or in the onset of hypertension (Figure 7.19b).

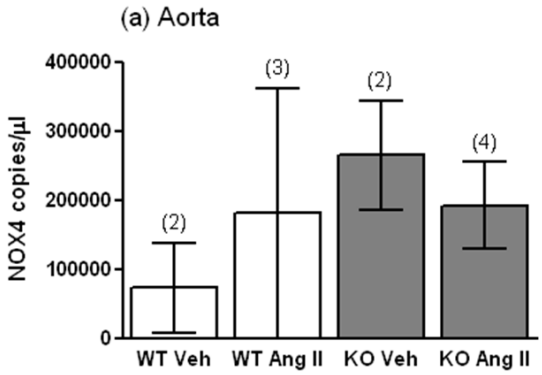
Relative NOX4 mRNA expression in the (a) aorta and (b) MRV and DRG in WT and αCGRP KO mice treated with either saline or Ang II for 28 days is shown in Figure 7.20. All results are expressed as copies/µl of cDNA. In the aorta (Figure 7.20a), when comparing WT and αCGRP KO vehicle treated mice no differences in expression were observed. In the onset of hypertension, again no differences in NOX4 mRNA expression were observed in both WT and αCGRP KO mice. With regards to the mesenteric resistance vessels and dorsal root ganglia, no difference in NOX4 mRNA expression was found in WT and αCGRP KO vehicle treated mice, or in the onset of hypertension (Figure 7.20b).



(b)

Organ	WT Veh	WT Ang II	KO Veh	KO Ang II
MRV	259±0.5	422±116	162±34	436±131
DRG	307±37	193±26	246±47	234±33

Figure 7.19 Effect of Ang II (0.9mg/kg/day for 28 days) compared with vehicle (saline) on NOX2 mRNA expression in WT and αCGRP KO mice. NOX2 mRNA expression in the (a) aorta and (b) mesenteric resistance vessels and dorsal root ganglia, measured by RT-qPCR, expressed as copies/µl and normalised to SDHA, HPRT-1 and PLA_2. **p<0.01 compared to vehicle treated animals. N=2-4.



(b)

Organ	WT Veh	WT Ang II	KO Veh	KO Ang II
MRV	$6.8x10^5 \pm 12.1x10^5$	$4.2x10^5 \pm 0.9x10^5$	$8.7x10^5 \pm 1.1x10^5$	$5.5x10^5 \pm 2.8x10^5$
DRG	$0.7x10^5 \pm 1.0x10^5$	$1.8x10^5 \pm 1.1x10^5$	$2.6x10^5 \pm 2.1x10^5$	$1.9x10^5 \pm 0.5x10^5$

Figure 7.20 Effect of Ang II (0.9mg/kg/day for 28 days) compared with vehicle (saline) on NOX4 mRNA expression in WT and αCGRP KO mice. NOX4 mRNA expression in the (a) aorta and (b) mesenteric resistance vessels and dorsal root ganglia, measured by RT-qPCR, expressed as copies/µl and normalised to SDHA, HPRT-1 and PLA_2. Statistical evaluation showed no difference between the treatment groups. N=2-4.

7.6 Summary

- WT and αCGRP KO mice developed progressive hypertension after 28 days Ang II infusion (0.9mg/kg/day) when compared to their vehicle treated controls, however this hypertensive response was exacerbated in αCGRP KO mice at day 28 of Ang II infusion in comparison to hypertensive WT mice.

- Echocardiography failed to identify any end point markers of cardiac hypertrophy or dysfunction in WT and αCGRP KO mice after Ang II infusion in comparison to their vehicle treated controls. Heart weights from hypertensive mice were not increased when compared to their matched vehicle treated controls.

- αCGRP mRNA expression was significantly upregulated in the aorta, MRV and DRG of WT mice in the progression of Ang II induced hypertension at day 28. Circulating plasma αCGRP was also increased in WT mice at day 28 following Ang II infusion when compared to WT vehicle treated mice.

- βCGRP mRNA expression was significantly elevated in the aorta of WT mice treated with Ang II compared to vehicle controls, however this elevation was absent in αCGRP KO mice.

- CLR and RAMP1 aortic mRNA expression was elevated in Ang II treated WTs but expression did not differ in αCGRP KOs.

- Aortic eNOS mRNA expression was significantly decreased and ET-1 mRNA was elevated in hypertensive WT and αCGRP KO mice following 28 days Ang II infusion.

- IL-6 and VCAM-1 mRNA was substantially elevated in the aortas of hypertensive WT mice at day 28 following Ang II infusion. These genes were also elevated in αCGRP KO mice, but at a blunted level in comparison to WTs.

- Vascular hypertrophy occurred in the onset of hypertension, characterised by increased SMC width in αCGRP KO mice following 28 days Ang II infusion.

- Collagen III mRNA expression was significantly elevated in the onset of hypertension in both WT and αCGRP KO mice. αCGRP KO mice however displayed exacerbated increases when compared to hypertensive WTs.

- HO-1, GPX and SOD1 aortic mRNA expression was significantly elevated in WT mice following Ang II infusion for 28 days when compared to their matched vehicle controls. However the αCGRP KO mouse only shows elevation of SOD1 following Ang II infusion, and not HO-1 or GPX.

- NOX 2 aortic mRNA is elevated in hypertensive WT and αCGRP KO mice following Ang II infusion, however no changes in NOX 4 mRNA are observed in WT and αCGRP KO mice at day 28 following Ang II infusion compared to their matched vehicle controls.

7.7 Discussion

The aim of this chapter was to identify a role for αCGRP in Ang II induced hypertension after 28 days and compare this to the 14 day study. We also wanted to identify whether the increased αCGRP expression was long term in the onset of hypertension induced by Ang II, or whether this expression was lost later on at day 28. The main results from this study are identified in the summary. We have demonstrated that αCGRP is still elevated at day 28 of Ang II infusion, therefore confirming that αCGRP does indeed play a longer term role in this disease progression, and cancels any initial questions as to whether this neuropeptide has only a short-acting role at the early steps in the onset of the hypertension. Again we have observed exacerbated hypertension in the αCGRP KO mice compared to WT mice. This is also characterised by progressed vascular hypertrophy, endothelial dysfunction and the elevation of inflammatory and oxidative stress markers. However whilst some results are similar at days 14 and 28, others differ as the hypertension progresses in both WT and αCGRP KO mice which will now be discussed further whereby a detailed comparison of both studies will be made. As previously discussed in the methods in Chapter two, the results from these studies were obtained from two independent experiments, therefore experimental variation may have occurred which may influence the copies expressed. With this in mind the results presented in this section are expressed as a fold change from their matched vehicle controls in order to accurately compare trends between the studies, and not copy number expression.

7.7.1 Blood Pressure

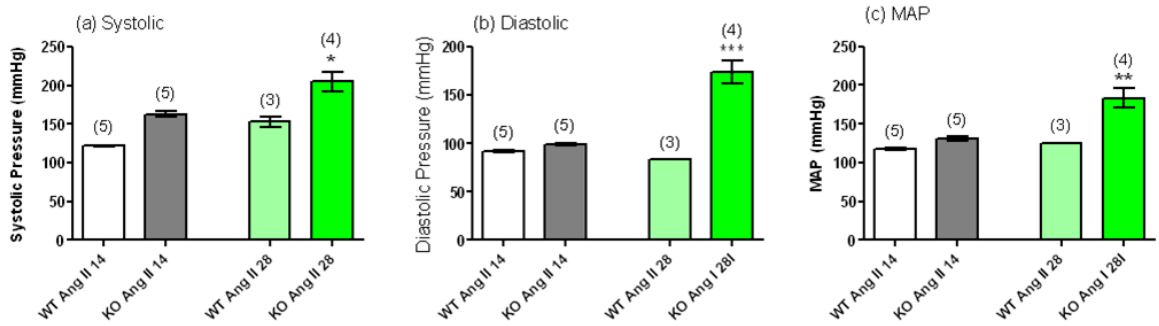


Figure 7.21. Comparing the effects of Ang II infusion for 14 days (1.1mg/kg/day) and 28 days (0.9mg/kg/day) on BP in WT and αCGRP KO mice. *(a) mean systolic BP, (b) mean diastolic BP and (c) mean arterial pressure (mmHg) in WT and αCGRP KO mice infused with Ang II for either 14 or 28 days. Statistical evaluation by one-tailed t-test where *p<0.05, **p<0.01 and ***p<0.001 compared to their matched genotype at 14 days. N=3-5.*

At day 14 both WT and αCGRP KO mice showed a significant increase in BP after Ang II infusion when compared to their matched vehicle controls. These measurements were within the mild-moderate hypertensive range and therefore we could confirm that 1.1mg/kg/day for 14 days caused the onset of hypertension in WT and αCGRP KO mice. However this hypertension was exacerbated in the absence of αCGRP, as shown in the αCGRP KO mice, therefore we suggested a potentially protective role for αCGRP in the onset of hypertension at 14 days (Figure 3.4). When we prolonged this infusion for 28 days to gain a model of established hypertension, BP in WT mice at day 28 did not differ significantly to that at day 14 as shown in Figure 7.21. This lack of increasing BP over time in WT mice has also been previously shown in a study by Jennings *et al.* 2012 who also used a time course study of 13 and 28 days Ang II infusion (700ng/kg /min) to look at renal function in C57BL/6 wildtype and Cyp1b1-/- mice. The reason behind them doing this was because at day 13 they observed renal dysfunction but not renal damage in WT mice, therefore they carried out a longer term study to look at end point renal damage. At day 13 MAP in Ang II treated WT mice recorded by

tail-cuff was 177±4mmHg compared to 105±3mmHg. When the time of infusion was lengthened, this BP did not change and at day 28 MAP in Ang II treated mice was 172±10mmHg, however renal damage was established in this model by day 28 (Jennings *et al.* 2012). We can therefore hypothesise that 14 days of Ang II treatment is enough to elevate BP and maintain hypertension at a steady level, however longer infusion will not enhance the BP, but will continue to cause remodelling and consequent end organ damage and inflammation. However this was not the case in the αCGRP KO mice, whereby BP continued to increase and by day 28 we observed a mean systolic pressure of approximately 200mmHg compared to 160mmHg observed at day 14 ($p<0.05$, Figure 7.21a). Diastolic pressure was also now significantly increased at day 28 in the CGRP KO mice compared to that at day 14 ($p<0.001$, Figure 7.21b). This was also true for MAP measurements ($p<0.01$, Figure 7.21c). We can therefore presume that in the absence of αCGRP, Ang II infusion causes BP to increase continuously to detrimental levels by day 28 in comparison to maintained BP between days 14-28 in WT mice, therefore providing a beneficial role for αCGRP in the maintenance of BP in both the onset of Ang II induced hypertension at day 14 and the established model of hypertension by day 28. However it is worth noting here that at day 14 the predominant increase in BP in αCGRP KO mice was systolic dependent, but at day 28 there are significant increases in both systolic and diastolic pressures. This suggests that at day 14 the mice were suffering from isolated systolic hypertension. As this hypertension progressed the raise in diastolic pressure indicates a more severe hypertensive state as this increase is suggested to be a marker of left ventricular dysfunction, or perhaps increased peripheral resistance (Yamamoto *et al.* 1996). Bivalacqua *et al.* have previously shown this beneficial role for CGRP in hypoxia induced hypertension whereby over expression of CGRP by gene transfer attenuated the elevation in BP in response to hypoxia induced hypertension over 16 days in mice compared to WT mice with no αCGRP over expression (Bivalacqua *et al.* 2002). Administration of CGRP has also been previously shown to attenuate the BP increases in rodent models of hypertension such as DOCA-salt, L-NAME-induced and in the spontaneously hypertensive rat as previously discussed in the general introduction. However although the role of CGRP in other models of hypertension is well documented, the work presented here in this thesis is novel as we are the first research group to our knowledge to

show the dramatic elevation in BP using these αCGRP KO mice in an Ang II model of hypertension when compared to the BP rise in WT mice. In addition to this, using a time dependent study we are also the first to report that the BP continues to rise in the αCGRP KO mouse from day 14 up to day 28. Our data strongly suggests an extension to previous reports. These results suggest that αCGRP plays a pivotal and potentially chronic role in both the onset and the subsequent established phase of Ang II induced hypertension, in this model at least.

7.7.2 α and βCGRP

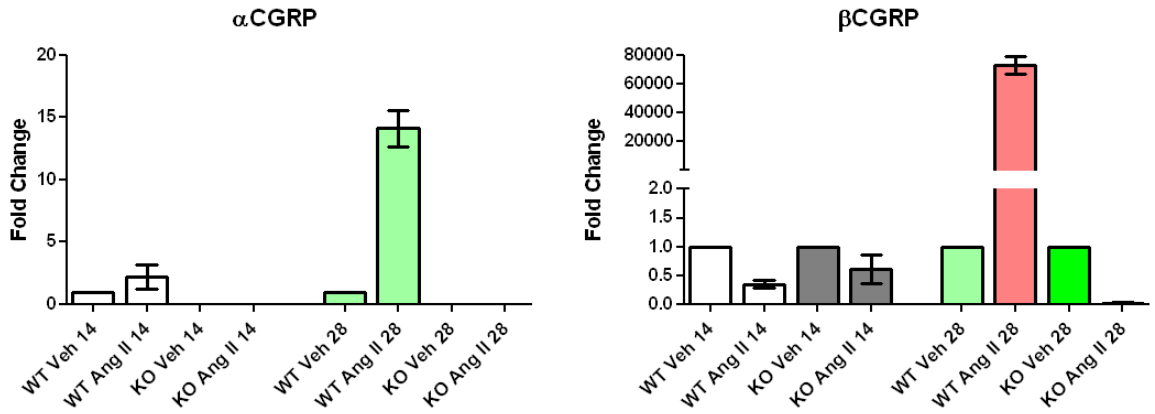


Figure 7.22. Comparing the effects of Ang II infusion for 14 days (1.1mg/kg/day) and 28 days (0.9mg/kg/day) on aortic αCGRP and βCGRP mRNA expression in WT and αCGRP KO mice. *Schematic representation of the average fold change in αCGRP and βCGRP expression in WT and αCGRP KO mice when compared to the average vehicle treated control expression after either 14 or 28 days Ang II infusion.*

In the onset of hypertension, I have shown that aortic tissue αCGRP mRNA expression was significantly increased by approximately 2 fold at day 14 in WT mice infused with Ang II (Figure 4.1). This expression was shown to still be apparent and elevated further by approximately 15 fold at day 28 when compared to their matched vehicle treated controls (Figure 7.4 and Figure 7.22). Circulating plasma αCGRP was also elevated in comparison to WT vehicle treated controls at day 28 (Figure 7.6). When comparing aortic βCGRP mRNA expression in both studies, at day 14 βCGRP expression was decreased approximately 0.5 fold in both WT and αCGRP KO mice after Ang II infusion (Figures 4.2 and 7.22). This decrease was also apparent at day 28 in αCGRP KO mice (Figures 7.5 and 7.22). However when comparing the data for Ang II treated WTs at day 14 and 28, although this expression was originally decreased at day 14, it is shown to be significantly increased by day 28. The lack of βCGRP expression in the αCGRP KO mice was unexpected. Although it is generally considered that the beta isoform is primarily expressed in the enteric nerves, it has also been shown to be expressed within the vasculature and biologically active in the nervous system.

For example Mulderry and co workers showed that βCGRP is found in the dorsal root ganglia and the spinal cord of rats (Mulderry *et al.* 1988). Holman *et al.* also reported βCGRP to be a coronary vasodilator in the rat (Holman *et al.* 1986). Brain and co workers also showed β CGRP to have similar vasodilatory actions to α CGRP in rabbit skin, in which they both increased blood flow and potentiated local bradykinin-induced oedema in a similar fashion (Brain *et al.* 1986). Systemic βCGRP has also been shown to decrease arterial BP in hypertensive dogs (Verburg *et al.* 1989) therefore suggesting a role for the beta isoform in hypertension also. In this study we have shown that βCGRP is expressed in the vasculature, including the aorta, with this expression being significantly more abundant than αCGRP. This βCGRP expression also continues to increase in WT mice from day 14 to 28 independently of an increase in BP which is novel data. The reason for the lack of the β isoform expression in the aorta of αCGRP KO mice at day 28 of Ang II infusion is unknown as these isoforms are derived from two distinct different genes and as such we originally thought that perhaps βCGRP would compensate for the absence of αCGRP in the progression of hypertension, which is not the case. We also see similar trends in terms of βCGRP expression in the DRG at both 14 and 28 days, however expression when measured by relative copy numbers indicates that the β isoform is more abundantly expressed compared to αCGRP expression. The β isoform is also more abundantly expressed in the aorta and the mesenteric resistance vessels. This suggests that the source of this expression is both neuronal and non-neuronal. We have already shown positive CGRP staining in the aorta (Figure 4.4) after 14 days Ang II. This antibody detected both α and β isoforms, however in the αCGRP KO mice, we still observe positive staining in the aorta, suggesting that this staining must be βCGRP specific, and this staining was located in all cell types of the aorta, therefore confirming that the source of this isoform is non-neuronal.

Increased CGRP protein expression in the aorta and plasma has been documented previously in models of vascular restinosis whereby CGRP expression was elevated by 64% in the aorta and 89% in the plasma compared to control groups (Liu *et al.* 2003). In addition to this, infusion of Ang II to normotensive humans has previously been shown to cause dose-dependent increases in plasma CGRP levels, in parallel with an increase in BP induced by

Ang II (Portaluppi *et al.* 1993). This data does fit in with our work to a certain extent; however our results show that as the hypertension progresses, CGRP expression continues to rise independently of an increase in BP. When reviewing the literature, in both rodent and human models of hypertension, CGRP levels in hypertensive subjects compared to controls still remain inconclusive with some studies reporting increased levels, whilst others report decreased or even unchanged CGRP levels in hypertension (See general introduction for in-depth review on this literature). Also the differing results observed in the literature may perhaps have been due to the time point by which CGRP was measured, i.e. CGRP levels may have been measured too late on in the progression of hypertension, and may have in fact been raised earlier on. Our data shows that αCGRP expression is raised in the Ang II model of hypertension. However what is novel about this data is that my study has answered our original question as to whether αCGRP would still be expressed in a model of longer term sustained hypertension. Due to the significant elevation from day 14 to day 28 we can presume that the actions of αCGRP are indeed more longer term and not acute which was originally questioned after the results obtained from the 14 day study. We have also shown that this further increase in αCGRP expression from day 14 up to day 28 is also independent of an increase in BP in WT mice. This has not been reported previously. Here, at 28 days the hypertension remained less severe at day 28 in the WT mice when compared to the αCGRP KOs. This study therefore provides further evidence for a potentially protective and therapeutic role of αCGRP in both the onset and progression of Ang II induced hypertension.

7.7.3 CLR and RAMP1

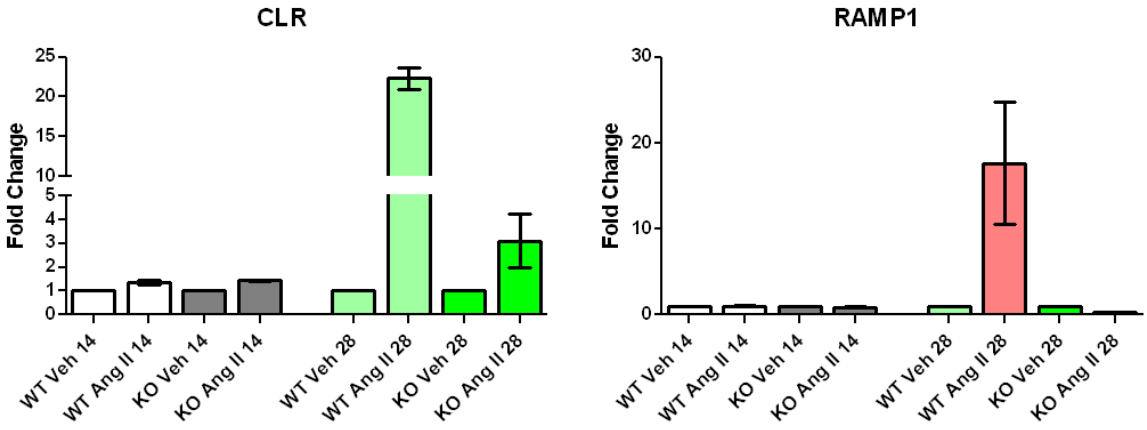


Figure 7.23. Comparing the effects of Ang II infusion for 14 days (1.1mg/kg/day) and 28 days (0.9mg/kg/day) on aortic CLR and RAMP1 mRNA expression in WT and αCGRP KO mice. *Schematic representation of the average fold change in CLR and RAMP1 expression in WT and αCGRP KO mice when compared to the average vehicle treated control expression after either 14 or 28 days Ang II infusion.*

In the onset of hypertension, at day 14 aortic CLR mRNA expression was significantly elevated by approximately 2 fold in both WT and αCGRP KO mice when compared to their vehicle treated controls, however this expression did not differ between the two genotypes (Figure 4.5a). This expression has continued to increase and by day 28, CLR mRNA was increased by approximately 20 fold in hypertensive WT mice in comparison to their matched vehicle controls (Figure 7.7a). This expression was also increased in the hypertensive αCGRP KO mice, however only by approximately 3 fold which is blunted in comparison to the increased expression observed in the WT mice at day 28 following Ang II infusion. RAMP1 mRNA expression did not differ in WT and αCGRP KO mice at day 14 following Ang II infusion (Figure 4.6a).This result did not differ either at day 28 in αCGRP KO mice (Figure 7.8a). Although not increased at day 14, by day 28 of

Ang II infusion RAMP1 expression was significantly elevated in WT mice by approximately 17 fold compared to their matched vehicle treated controls (Figure 7.23). The continuous increase in CLR and RAMP1 expression in the aorta fits in nicely with the increasing α and β CGRP expression in hypertensive WT mice at day 28. In terms of fitting this data in with that previously shown in the literature, CGRP and its receptor components expression have been reported to be increased in several models of hypertension including Ang II (Li and Wang, 2005), human (Portaluppi *et al*. 1993), SHR (Pan *et al.* 2006) and Mineralocorticoid-salt (Supowit *et al.* 1995). In the Ang II model, Li and Wang showed that 10 days Ang II infusion into Wister rats significantly increased BP compared to vehicle controls, however CGRP administration was able to inhibit this MAP increase, whilst the CGRP antagonist $CGRP_{8-37}$ caused a further increased in MAP compared to Ang II infusion alone. Plasma and DRG CGRP levels did not differ amongst the groups, however CLR and RAMP1 mRNA expression was significantly upregulated in the mesenteric arteries of Ang II treated rats (Li and Wang, 2005). In addition to this, more recently, over expression of RAMP1 was reported to attenuate Ang II-induced hypertension compared to WT mice (Sabharwal *et al*. 2010).

In terms of receptor blocking, in normotensive subjects pharmacological blockade of CGRP receptors does not influence MAP. However contrary to this, in experimental models, receptor blockade increases the severity of hypertension (Wang and Wang, 2004; Supowit *et al.* 1997). Supowit and co workers used a DOCA-salt model of hypertension in the rat to study this whereby they induced DOCA-salt hypertension in the Sprague-Dawley rats for 4 weeks which resulted in a significant increase in MAP compared to normotensive controls. Induction of the CGRP and receptor antagonist $CGRP_{8-37}$ in normotensive rats had no effect on MAP, however a rapid increase in the already elevated MAP was observed when the antagonist was given to the already hypertensive rats therefore indicating a protective role for CGRP in hypertension. Based on these findings, it is thought that endogenous CGRP operates through a negative-feedback mechanism in order to oppose the development of hypertension (Sabharwal *et al.* 2010). Upregulation of CLR and RAMP1 expression was reported in mesenteric vessels of Ang II induced hypertensive rats as previously discussed (Li and Wang, 2005). However they did not find any differences in circulating plasma CGRP levels after

10 days. They concluded that this increase in receptor expression was pressure dependent; however our study has shown that this increase is independent of an increase in BP. There is a gap in the literature with no evidence to demonstrate whether CGRP is able to regulate its own receptor expression, or whether it relies on an external stimulus, therefore this is an area of research which requires development of knowledge. The elevating response of CGRP and its receptor components may instead be in line with the inflammation and hypertrophy which continues to increase from day 14 to 28 of Ang II infusion, independently of an increase in arterial pressure.

7.7.4 Potential major vasoactive mediators eNOS (source of NO) and ET-1

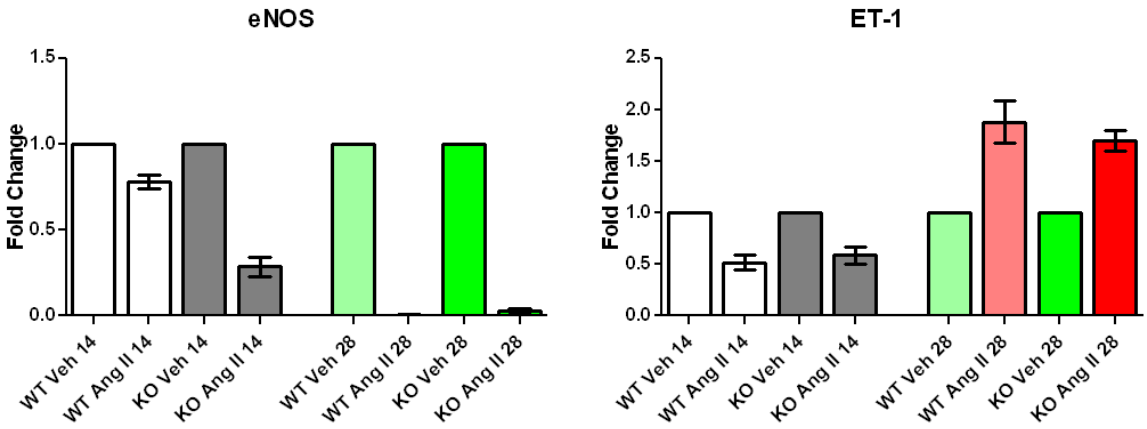


Figure 7.24. Comparing the effects of Ang II infusion for 14 days (1.1mg/kg/day) and 28 days (0.9mg/kg/day) on aortic eNOS and ET-1 mRNA expression in WT and αCGRP KO mice. *Schematic representation of the average fold change in eNOS and ET-1 expression in WT and αCGRP KO mice when compared to the average vehicle treated control expression after either 14 or 28 days Ang II infusion.*

NO derived from the endothelium is an important molecule in the regulation of vascular tone via relaxation of the vascular smooth muscle cells and subsequent vasodilatation (Vane and Botting, 1992). This NO also has anti-atherogenic effects including the inhibition of VSMC proliferation and platelet aggregation. Inhibition of endothelial NO can be detrimental as this loss results in endothelial dysfunction which has been observed in patients with coronary risk factors such as hypertension and hypercholesterolemia. This loss is also associated with increased oxidative stress and superoxide radical production, therefore making the role of NO in the vasculature vital in preserving health and a normal physiological state (Botting *et al.* 1989).

In the onset of hypertension induced by Ang II, at day 14 aortic eNOS mRNA expression was significantly decreased in both WT and αCGRP KO mice when compared to their matched vehicle controls, however this expression was significantly lower in αCGRP KO mice, suggesting a larger degree of inhibited NO

bioavailability in the absence of αCGRP as hypertension progresses (Figure 5.1a). By day 28 this eNOS expression is equally diminished in hypertensive WT and αCGRP KO mice (Figures 7.9a and 7.24).

When comparing aortic ET-1 mRNA expression, although not significant, at day 14 both hypertensive WT and αCGRP KO mice displayed a trend of decreasing ET-1 expression after Ang II infusion (Figures 5.2a and 7,24). By day 28 this trend has changed and ET-1 expression was now increased in both Ang II treated WT and αCGRP KO mice compared to their matched vehicle controls, however there was no difference in ET-1 expression at day 28 when comparing the hypertensive WT and αCGRP KO mice (Figures 7.10a and 7,24).

The role of eNOS in hypertension was once controversial. In mice, hypoxia was associated with increases in lung eNOS (Fagan *et al.* 2001). This increase in eNOS was also reported in the rat lung during hypoxia (LeCras *et al.* 1998) and in pulmonary hypertension (Tyler *et al.* 1999; Resta *et al.* 1999). In contrast to this, the loss of eNOS was also shown to result in systemic hypertension in eNOS -/- mice (Huang *et al.* 1995), with the progression of hypertension also being more severe in these KOs after treatment with L-NAME for 8 weeks (Knowles *et al.* 2000). Moreover, eNOS was also shown to serve important basal regulatory functions in the vasculature (Knowles *et al.* 2000). A study by Kojda *et al.* using eNOS KO and transgenic mice showed eNOS KO mice to be mildly hypertensive under basal conditions, whereas eNOS heterozygous mice were normotensive and showed no difference in basal BP in comparison to normotensive WT controls (Kojda *et al.* 1998). It was also shown by Ohashi *et al.* that over expression of eNOS causes hypotension (Ohashi *et al.* 1998). In light of this conflicting data, more recently a study by Gu *et al.* has suggested the expression of eNOS in hypertension to be time-dependent. In their study the expression of eNOS was significantly decreased in the glomeruli and arterioles/small arteries of patients with malignant hypertension, however no changes in eNOS expression was reported in patients with mild hypertension, suggesting that this reduction in expression is time dependent, and that subjects have a time-dependent compensatory mechanism which is lost as the hypertension progresses due to lack of vasodilatory mechanisms by NO production and increasing vasoconstrictor release (Gu *et al.* 2010). In this thesis I have shown that at 14 days of Ang II

infusion eNOS expression is decreased, which fits in with the elevated BP. As the hypertension progresses, this expression decreases further and it is virtually abolished by day 28. This data also fits in with that of Xue and Johns (1995) and Uren *et al.* (1992) who also reported significantly decreased eNOS protein expression in patients with pulmonary hypertension. At day 14 the eNOS expression in αCGRP KO mice was significantly lower than that of WT mice in the onset of Ang II induced hypertension. This result could suggest that either a) αCGRP is protecting against the hypertension progression, or b) CGRP is protecting against the loss of NO at day 14, however this mechanism is lost by day 28 as there are no differences in eNOS expression in the developed model of Ang II induced hypertension when comparing expression in WT and αCGRP KO mice, suggesting that there may be a time-dependent mechanism/relationship. It is also possible that the increase in BP is also due to a result of the loss of NO derived from eNOS, and not just, or even in part from the vasodilator αCGRP. CGRP is known to cooperate with NO in a tissue specific manner and can induce its vasodilatation via NO-dependent and independent mechanisms. When dependent on NO, CGRP stimulates adenylate cyclase and guanylate cyclase to induce vasorelaxation via NO release (Brain and Grant, 2004). There therefore may be a lack of this mechanism in this model.

At day 14 we initially hypothesised ET-1 to be raised in this model in both WT and αCGRP KO mice based on previous literature reporting increased ET-1 expression in Ang II induced hypertension, including that of Rajagopalan *et al.* who reported ET-1 expression to be significantly increased in the VSMCs of rats after 5 days Ang II infusion at a lower dose than used in our study (0.7mg/kg/day, Rajagopalan *et al*. 1997). However this finding was not observed in our study. By comparison, at day 28 we observed raised ET-1 expression in the aortas of WT and αCGRP KO mice, perhaps suggesting that the increase in ET-1 expression was time-dependent but the changes are due to a secondary, rather than a primary mechanism after Ang II-induced hypertension. A study by Jun An *et al.* showed that the release of ET-1 in response to Ang II is both concentration and time-dependent in cultured adventitial fibroblasts. In this study Ang II evoked a concentration- and time-dependent increase in ET-1 mRNA expression with increases in mRNA being evident within 30 min after Ang II treatment and this expression peaking within 1.5 hours (Jun An *et al.* 2006). With this literature in

mind, we can assume that protective mechanisms meant that 14 days was not long enough for ET-1 expression to be raised *in vivo*. By day 28 ET-1 expression was raised, however the lack of difference in expression between WT and αCGRP KO mice suggests that αCGRP does not play a role in ET-1 expression in this Ang II induced model of hypertension. In contrast to our *in vivo* study, recent studies have proposed a direct interaction between ET-1 and CGRP primarily due to ET_A receptors being expressed on sensory motor nerves alongside CGRP (Wang *et al.* 2006), which in turn has been shown to activate adenylate cyclase (AC, Brain and Grant, 2004). Studies by Joe De Meys research group in the Netherlands have shown that ET-1 induced arterial contractions were reversed and therefore sensitive to relaxation by the administration of CGRP but not sodium nitroprusside in a specific manner (Meens *et al.* 2009; Meens *et al.* 2011). This group have also reported ET-1 and CGRP expression to be elevated in cardiac and renal tissue in the SHR (Nelissen *et al.* 2011). It is possible that CGRP is combating ET-1 responses at 28 days and this is one reason why CGRP deletion is associated with the enhanced hypertension.

7.7.5 Vascular hypertrophy

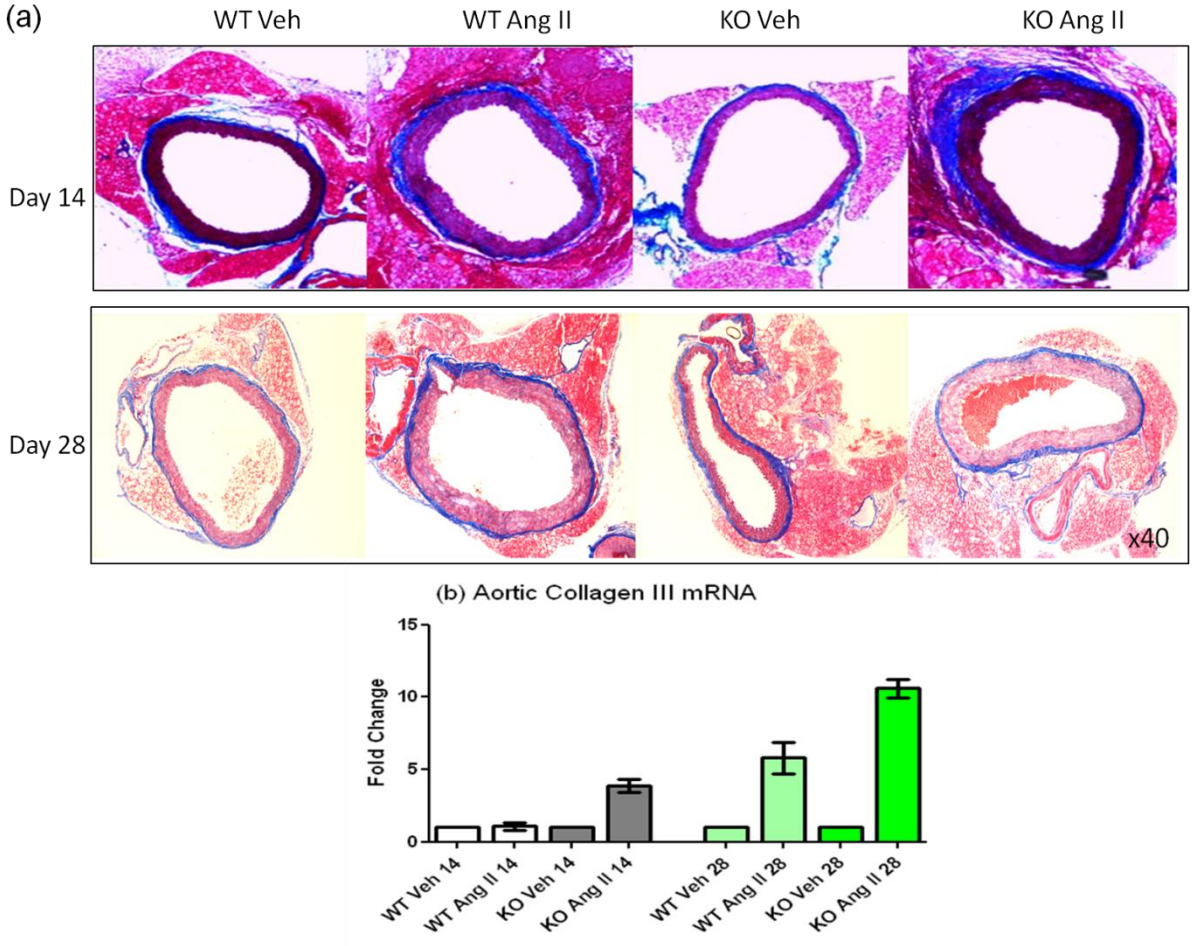


Figure 7.25. Comparing the effects of Ang II infusion for 14 days (1.1mg/kg/day) and 28 days (0.9mg/kg/day) on aortic hypertrophy/remodelling and Collagen III expression in WT and αCGRP KO mice. *(a) Representative images of aortic walls stained with Masson's trichrome at 40x magnification from WT and αCGRP KO mice treated with either vehicle or Ang II for 14 and 28 days. Figure combined from figures 5.10 and 7.13 (b) Schematic representation of the average fold change in aortic Collagen III expression in WT and αCGRP KO mice when compared to the average vehicle treated control expression after either 14 or 28 days Ang II infusion.*

Hypertension is a condition associated with progressive structural changes in both elastic and muscular vessels characterised by increases in vessel wall diameter

and thickening of the intima media (Bund and Lee 2003; Intengan and Schiffrin 2001; Sasamura *et al.* 2005). In essential hypertension, the vessels exhibit a reduced lumen diameter, increased ratio of media thickness/lumen diameter and unchanged cross-sectional area of the media. As the hypertension progresses, moderate hypertension leads to a rearrangement of the vascular structure leaving the cross-sectional area of the arterial wall unchanged, reducing the lumen area. Severe hypertension results in hypertrophy of the vascular wall which results in an increase in the arterial wall cross-sectional area together with a decrease in lumen diameter. Due to this remodelling, the wall stress is elevated, and this in turn is followed by an increase in elastin and collagen synthesis and vascular stiffening (Deyl *et al.* 1987; Durmowicz and Stenmark 1999; Spector *et al.* 1978).

In our model of hypertension, at day 14 of Ang II treatment, vascular hypertrophy of the aorta was apparent in WT and αCGRP KO mice. When SMC and collagen measurements were taken, results showed that αCGRP KO mice displayed exacerbated vascular hypertrophy in comparison to the WT, characterised by increased collagen, but not SMC measurements (Figure 5.10-12). When we analysed aortic mRNA collagen III expression, the results also confirmed the histology data whereby collagen III expression was elevated in the onset of hypertension, however this expression was more profound in the αCGRP KO mice, suggesting an increased incidence of vascular hypertrophy at day 14 in accordance with the exacerbated BP (Figures 5.13 and 7,25). However these results were not observed at day 28 following Ang II infusion. At this time-point we again showed mean aortic wall measurements to be increased in WTs and significantly increased in αCGRP KO mice at day 28 following Ang II infusion, however this time the significant difference was found in VSMC measurements and not collagen (Figures 7.13-14). When assessing the aortic collagen III mRNA expression at day 28 however, αCGRP KO mice treated with Ang II still displayed a significantly elevated level of collagen III expression in comparison to the increasing expression observed in hypertensive WT mice (Figures 7.15 and 7.25), perhaps indicative of an increase in vascular stiffness (Graham *et al.* 2011). In a renovascular model of hypertension in dogs, surgical occlusion of the renal artery for two months resulted in increased hypertension, substantial aortic hypertrophy and aortic stiffness and resistance to stretch due to the substantial increase collagen expression (Fischer *et al.* 2003). The reason for the differences in results

at day 14 and day 28 could be due to a number of reasons. To our knowledge, it has not been documented previously as to whether collagen formation occurs prior to SMC proliferation in the development of hypertension; therefore the results of this study may perhaps provide evidence of this. As vascular remodelling progresses in Ang II induced hypertension, there is a marked reduction in lumen diameter which causes the BP to increase (Zhang *et al.* 2010). However in this study we did not measure the diameter of the vessel and only measured the thickness of the walls. The reason being that the aortas were not perfused to fix the vessel and therefore the measurement of diameter in this model would not be accurate as the vessel may perhaps be collapsed. This is a limitation of this study and perfusion of the vessel would be ideal in future experiments in order to gain more accurate measurements of vessel hypertrophy. Another reason for the differing changes at each time-point may simply be due to the remodelling occurring in the aorta at the early phase and not progressively worsening over a longer period of time. In the later developing stages of hypertension, it seems reasonable to suggest that the remodelling may be occurring in the resistance vessels rather than the coronary vessels, therefore it would deem useful to look at vessels such as the mesenteric resistance vessels in future experiments of long term Ang II infusion. The remodelling of the smaller resistance vessels are thought to have more profound dramatic effects on BP compared to the aorta as they are the dominant contributors to flow resistance (Jain *et al.* 2003) and may be linked to the increase in diastolic pressure, therefore this may be the reason for the absence of increased hypertrophy of the aorta at day 28, but an ever increasing BP, both systolic and diastolic in the αCGRP KO mice.

The data shown here identifies a protective role for αCGRP in inhibiting the progression of vascular remodelling in response to an increase in BP by Ang II infusion. The protective role of CGRP in resisting this remodelling has been documented previously whereby over expression of RAMP1 resulted in an increase in CGRP expression which subsequently protected against endothelial dysfunction via CGRP endothelial dependent vasodilatation in Ang II treated carotid and basilar arteries *in vitro* (Chrissobolis et al. 2010). Endothelial progenitor cells (EPCs) are a population of rare cells made predominantly in the bone marrow which can be released into the circulation and migrate to the site of

vascular injury where they differentiate into endothelial cells to enhance repair (Urbich *et al.* 2004). EPC derived CGRP has been shown to be protective against EPC senescence induced by Ang II (Zhou *et al.* 2010). To further emphasise the protective importance of EPC derived CGRP, VSMC hypertrophy has also been shown to be attenuated in Ang II induced hypertension (Fang *et al.* 2011). This inhibitory effect of CGRP in VSMC proliferation has also been shown in *in-vitro* studies using the Ang II stimulus (Qin *et al.* 2004). Furthermore, when administering CGRP derived EPCs to rats, lowered vascular resistance resulted alongside inhibited VSMC proliferation and thickening of the vessel wall (Zhao *et al.* 2007). Kochova *et al.* also reported increasing CGRP levels in the plasma and aorta in accordance to the elevating collagen expression associated with vascular remodelling in subtotally nephrectomized hypertensive rats after 10 days and 10 weeks (Kochova *et al*. 2009). In hypertension models using the αCGRP KO mice, increased vasculitis has previously been reported in the hearts of αCGRP KO mice in the DOC-salt induced model of hypertension with markedly increased thickening and inflammation of the vessel wall in comparison to that observed in WT mice. However it is worth noting that these KO mice have an increased basal BP and slight increase in heart to body weight ratio compared to WTs which may have contributed to some of the significant differences observed (Bowers *et al.* 2005).

We consider that the loss of αCGRP in our study contributed to an increase in collagen at day 14, and then an increase in SMC proliferation at day 28, therefore suggesting a protective relationship between αCGRP and the inhibition of vascular remodelling and collagen formation. These studies provide strong evidence for a protective role for αCGRP in the onset of hypertension and vascular remodelling.

7.7.6 Vascular inflammation markers

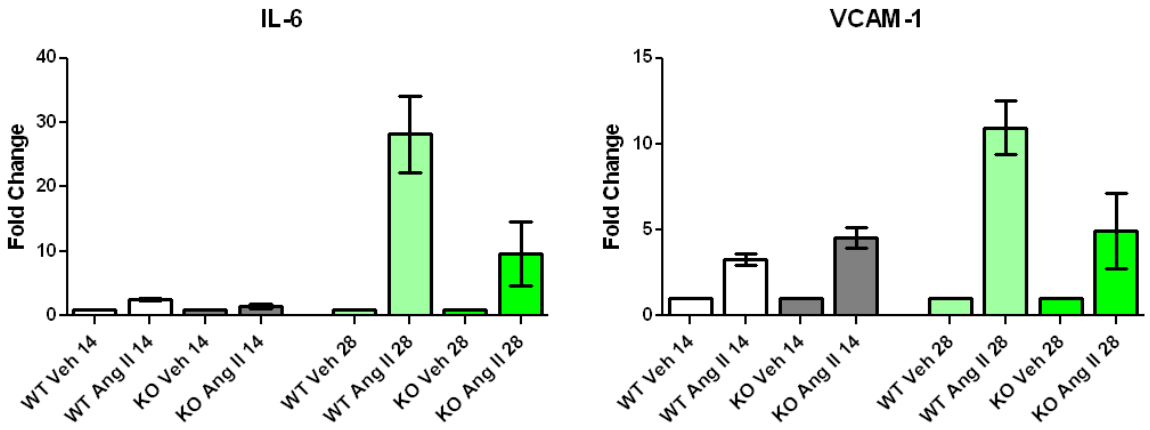


Figure 7.26. Comparing the effects of Ang II infusion for 14 days (1.1mg/kg/day) and 28 days (0.9mg/kg/day) on aortic IL-6 and VCAM-1 mRNA expression in WT and αCGRP KO mice. *Schematic representation of the average fold change in IL-6 and VCAM-1 expression in WT and αCGRP KO mice when compared to the average vehicle treated control expression after either 14 or 28 days Ang II infusion.*

When comparing aortic IL-6 mRNA expression at 14 and 28 days, IL-6 expression was significantly upregulated in the onset of Ang II induced hypertension after 14 days in WT mice. This expression was also upregulated in hypertensive αCGRP KO mice, although this was not significant (Figures 5.8a and 7.26). At day 28, this IL-6 mRNA expression was significantly elevated further in hypertensive WT mice, with an average of around 30 fold increase when compared to the vehicle controls at day 28. αCGRP KO mice also had increasing IL-6 expression at day 28 (~10 fold), however again this was not significantly more when compared to the αCGRP KO vehicle control group (Figures 7.11a and 7.26).

VCAM-1 was significantly elevated in the onset of hypertension in WT and αCGRP KO mice after 14 days Ang II infusion. This expression was significantly enhanced in the αCGRP KO mice when compared to WT mice at day 14 (Figures 5.3 and 7.26). After 28 days Ang II infusion, VCAM-1 expression was still elevated in Ang II treated mice, this being approximately 10 fold more in WT mice compared to that at 14 days. In αCGRP KO mice this expression was still

elevated at day 28 however there was no increase in this expression compared to that observed at day 14 (Figures 7.12a and 7.26). This comparison shows that after Ang II infusion at day 14, the absence of αCGRP causes exacerbated VCAM-1 expression when compared to hypertensive WT mice, suggesting a protective role for CGRP in the onset of VCAM-1 expression, however this cannot be said at day 28 as hypertensive WT mice are displaying a higher incidence of VCAM-1 expression compared to the αCGRP KO mice.

In terms of vascular inflammatory mediators in the progression of hypertension, CGRP activated endothelial cells have been shown previously to significantly reduce the number of inflammatory cells such as monocytes and neutrophils which were recruited to the vessel wall. Migration into the vessel wall and differentiation was also shown to be attenuated (Huang *et al.* 2011). Pre-treatment with CGRP protected mice from LPS-induced liver injury via the inhibition of pro-inflammatory cytokine production including IL-6 (Kroeger *et al.* 2009). In addition to this, Ang II infusion for 3 hours has been shown to increase IL-6 production in humans (Luther *et al.* 2006) and the hypertension induced by IL-6 is attenuated in IL-6 KO mice (Lee *et al.* 2006), therefore suggesting IL-6 to contribute significantly in the development of Ang II induced hypertension. In our model, whilst we have shown IL-6 expression to be significantly more upregulated in plasma of hypertensive KO mice compared to WT mice at day 14 suggesting a protective role for αCGRP, this is not observed in regards to tissue expression whereby there is no difference in aortic IL-6 expression at day 14. In addition to this, although IL-6 expression continues to increase at day 28 in the aorta of αCGRP KO mice, the elevation is more profound in the WT mice. The reason behind this is unclear. The protective role of αCGRP in IL-6 inhibition may perhaps be tissue specific, or else the relationship between CGRP and IL-6 inhibition may be time-dependent, however at this stage there is to our knowledge no evidence in the literature to support this. One possibility is that the IL-6 and VCAM-1 rises are seen earlier in the αCGRP KO mouse and have diminished by 28 days as the disease process accelerates.

CGRP expression suppresses the development of allograft vasculopathy by lymphocytes and inflammatory cells, marked by a reduction in VCAM-1 expression and inhibition of apoptosis induced by iNOS (Zhang *et al.* 2009). This

protective role has also been reported in mouse models of dermatitis with increasing levels of CGRP being reported in response to the increased leukocyte recruitment to the site of injury (Goebeler *et al.* 1994). More recently Huang *et al.* (2011) showed treatment of LPS-stimulated HMEC-1 cells with CGRP to inhibit the ability to chemoattract human neutrophils and mononuclear cells. In studies using the αCGRP KO mouse, it has been reported by Bowers *et al.* (2005) that the absence of αCGRP in DOCA-salt hypertension results in enhanced oxidative stress, inflammation, and renal histopathological damage with markedly time-dependent increases in VCAM-1, MCP-1 and ICAM-1 expression in the kidney compared to WT mice at 14 and 21 days. This mouse does show an increased basal BP compared to WT mice, however under basal conditions there was no difference in renal morphology. Thus, sensory nerves, via αCGRP, appear to be renoprotective against hypertension-induced damage (Bowers *et al.* 2005). This work is of similarity to ours whereby we showed αCGRP KO mice to have a higher degree of VCAM-1 expression in the aorta compared to that in WT mice at day 14. However as shown, this does not increase further over a longer infusion time, unlike the WT mice who show a continuing elevation of VCAM-1 expression at day 28. This result is similar to that of IL-6 expression, again suggesting that the protective role of αCGRP in inhibiting this inflammatory cascade and recruitment of cells to the site of injury may be time-dependent in this model and that deletion of αCGRP leads to an accelerated time course with the inflammatory phase peaking earlier. This suggestion would correspond to the increasing BP observed at day 28 in the αCGRP KO mice compared to that in the WTs. In addition to this, αCGRP expression is continuing to rise in WT mice independently of an increase in BP, suggesting that this vasodilator is still actively trying to inhibit this inflammatory response at day 28 of Ang II infusion in WT mice. We would therefore have hypothesised the inflammatory expression to be progressively increased in the αCGRP KO mice simply due to the lack of αCGRP and CGRP receptor expression. The studies mentioned provide evidence for a protective role for αCGRP in mediating an anti-inflammatory pathway, therefore giving rise to new avenues for therapeutic treatment of inflammatory conditions.

7.7.7 Endogenous anti-oxidants

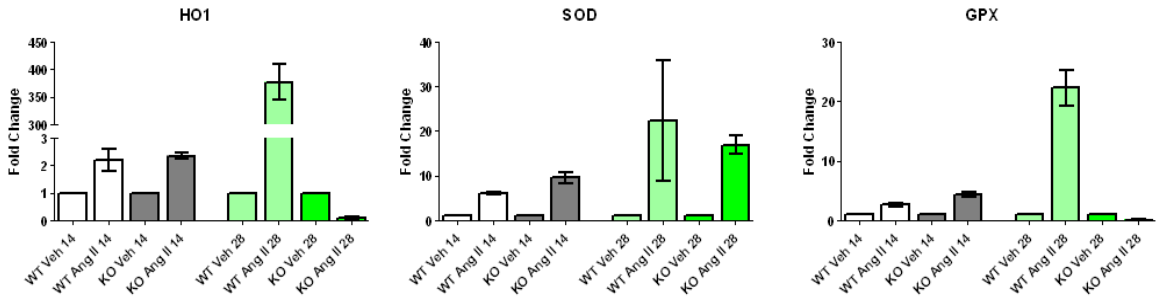


Figure 7.27. Comparing the effects of Ang II infusion for 14 days (1.1mg/kg/day) and 28 days (0.9mg/kg/day) on aortic HO-1, SOD1 and GPX mRNA expression in WT and αCGRP KO mice. *Schematic representation of the average fold change in HO-1, SOD1 and GPX expression in WT and αCGRP KO mice when compared to the average vehicle treated control expression after either 14 or 28 days Ang II infusion.*

At day 14, HO-1 mRNA expression did not differ between vehicle treated animals. This was also true for vehicle treated WT and αCGRP KO mice after 28 days. In the onset of hypertension, after 14 days HO-1 mRNA expression was upregulated in WT mice (Figures 6.1a and 7.27). At day 28 this increase was elevated further in comparison to the vehicle control (Figures 7.16a and 7.27). In αCGRP KO mice, at day 14 HO-1 mRNA expression was significantly upregulated in the onset of hypertension, however by day 28 this expression was lost, suggesting a loss of the generation of antioxidant levels at this time point (Figures 6.1a, 7.16a and 7.27).

SOD1 mRNA expression did not differ between WT and αCGRP KO mice at day 14 and 28 of saline infusion. SOD1 mRNA expression was significantly raised in the onset of hypertension at day 14 in WT mice, and continued to rise as shown at day 28. In αCGRP KO mice, SOD1 mRNA was significantly elevated in the onset of hypertension at day 14, and this increase was significantly higher than that of

Ang II treated WT mice (Figures 6.3a and 7.27). This expression continued to rise as the disease progressed and at day 28 SOD1 expression was elevated further. However at day 28 there was no difference in SOD1 mRNA expression when comparing the two hypertensive groups (Figures 7.17a and 7.27).

GPX mRNA expression did not differ between vehicle treated WT and αCGRP KO mice at day 14. At day 28, vehicle treated αCGRP KO mice appear to have increased GPX mRNA expression compared to WT mice; however this is not significant and could be due to experimental error or low n numbers. In the onset of Ang II induced hypertension, at day 14, GPX mRNA expression is upregulated in WT mice (Figures 6.2a and 7.27). This expression continues to increase and is increasingly elevated by day 28 (Figures 7.18a and 7.27). This increase was also observed in αCGRP KO mice at day 14, however by day 28 this expression is lost, similar to what was observed in terms of HO-1 expression. It therefore seems reasonable to presume that at day 14, in the absence of αCGRP the antioxidant genes are actively being produced in order to counteract the exacerbated hypertension. However by day 28 the antioxidant defence system is overwhelmed and can no longer fight against the increasing BP and inflammatory response, which in turn leads to end point vascular damage.

The protective role of the antioxidants HO-1, SOD1 and GPX in the onset of hypertension have been documented previously. HO-1 knockout mice results in increased production of pro-inflammatory cytokines (Kapturczak *et al.* 2004) whilst overexpression of HO-1 in VSMC inhibits cardiovascular damage induced after 14 days Ang infusion II in HO-1 transgenic mice (Morita *et al.* 2005). HO-1 induction prevented the development of Ang II induced hypertension and lowered BP in the established Ang II hypertension (Vera *et al.* 2007; Yang *et al*. 2004). Evidence for a long-term control of BP by HO-1 was shown by Wang *et al.* 2006 who demonstrated sustained lowering of BP in SHRs using a HO-1 inducer hemin which was infused via a minipump for 3 weeks (Wang *et al.* 2006).

In terms of SOD1, aortic rings from SOD1 KO mice display impaired endothelial dependent relaxation compared to WT mice (Didion *et al*. 2002). In a mouse model of hypertension, elevated SOD1 expression and decreased superoxide production was reported previously in mice infused with Ang II (Wang *et al.* 2002)

therefore suggesting that SOD1 serves an important role in the development of hypertension.

In hypertension, treatment with ACE inhibitors improves endothelial dysfunction and delays the progression of atherosclerosis through oxidative stress inhibition (Khan *et al.* 2004), antioxidant elevation (Moinudddin *et al.* 2011) and increased NO bioavailability (Mancini *et al.* 1996). Recent studies have suggested that the mechanisms by which these drugs do this are thought to be through upregulation of the glutathione-dependent antioxidant defences (De Cavanagh *et al.* 1999; De Cavanagh *et al*. 2000). Oxidative stress is exacerbated in the GPX deficient mouse compared to WT's (Gao *et al.* 2010) therefore suggests a protective role for GPX in combating against oxidative stress in the onset of hypertension and vascular related diseases.

In this study we have shown that gene expression of all three antioxidants is elevated in WT mice during the onset of Ang II induced hypertension at day 14. This fits in with the previously reported literature in response to the elevation in BP. As the hypertension progresses, the expression of these antioxidants continue to rise further in WT mice although there is no further elevation in BP. This suggests that the rise of antioxidant levels is independent of an increase in BP, and is likely to be in response to the increasing IL-6, VCAM and ET-1 levels in these mice, in which the release of antioxidants will try and fight against the rising inflammation. Also the decrease in eNOS expression may have caused a further elevation in endogenous antioxidant enzyme levels, which has been previously reported by Turkseven *et al.* (2005) whereby increased HO-1 expression resulted in a elevation back to the normal level of previously decreased eNOS expression in diabetic rats. Importantly, here, this may be due to the increase in CGRP expression, as it has been reported previously that CGRP gene therapy suppresses ROS generation by enhancing SOD-1 in diabetic mice (She *et al.* 2003; Li *et al.* 2012), therefore the increase in CGRP may be directly related to an increase in antioxidant levels.

In the αCGRP KO mice, antioxidant expression was also elevated at day 14 following Ang II infusion, however the expression on SOD1 and GPX was of a higher degree than that in the WT mice. These mice displayed exacerbated hypertension and vascular remodelling, therefore the increased expression of

antioxidants observed in these mice was a sign that the lack of αCGRP resulted in the antioxidant defence system having to fight harder to reduce the elevated BP. However when we observed the antioxidant expression at day 28, apart from GPX, antioxidant expression was lost. When comparing this to other studies conflicting results have been reported in terms of HO-1 expression in the hypertensive state in rats and humans, some showing HO-1 to be enhanced (Ishizaka *et al.* 1997; Ishizaka *et al.* 2005), whilst others report HO-1 to be decreased (Ndisang and Wang, 2003; Makino *et al.* 2001). It was suggested by Alba *et al.* that Ang II may decrease HO-1 expression in the long term progression of hypertension, thus losing the antioxidants protective properties (Alba *et al.* 2008). The same study also reported depressed HO-1 expression in neutrophils from moderate-severe hypertensive patients compared to healthy patients. In addition, Ang II treated cultured human neutrophils resulted in reduced HO-1 enzyme activity (Alba *et al*. 2008). However in contrast to this, Cheng *et al.* (2004) carried out a study to observe the time-course changes of HO-1 induction in aorta during the development of hypertension in the SHR. They found that at 8 weeks, HO-1 expression was induced at the onset of hypertension, and this level was progressively maintained up until the end of the experiment at week 16 (Cheng *et al.* 2004). In a rat model of severe pulmonary hypertension, HO-1 was found to be localised in endothelial and VSMC and loss or decreased expression of this HO-1 in the pulmonary hypertensive lesion resulted in a further progression of vascular hypertrophy (Achcar *et al.* 2005). SOD and GPX expression has also been shown to be decreased in the progression of hypertension, whereby decreases in SOD and GPX occurs in red blood cell membranes of uncontrolled hypertension. However when this hypertension is treated, these levels revert back to normal (Das, 2006; Kumar *et al.* 1993). We already know that an increase in CGRP expression through gene therapy results in an increase in vasodilatation and expression of antioxidants (She *et al.* 2003); therefore the loss of αCGRP in these mice suggests a direct link with the lack of antioxidant expression at day 28. The initial rise in antioxidants at day 14 seems to have been an attempt to try and fight against the rise in BP and inflammation. However by day 28, the further increase in BP and lack of αCGRP is overwhelming and we can presume that the antioxidant defence system can no longer actively fight against this. With this in mind we can assume that in absence of this defence, these mice are in fact

increasingly stressed with an increasing progression of severe, and potentially life threatening state of hypertension. The further increase in GPX expression in these mice is unknown. Wang *et al.* (2007) have reported a decrease in SOD whilst GPX is increased in pre-hypertensive patients with decreased CGRP levels compared to normotensive subjects; however it is unclear as to why this occurs, with no other literature to support this, but this is not dissimilar to what we have shown here in the αCGRP KO mice at 28 days (Wang *et al.* 2007).

7.7.8 NADPH oxidases

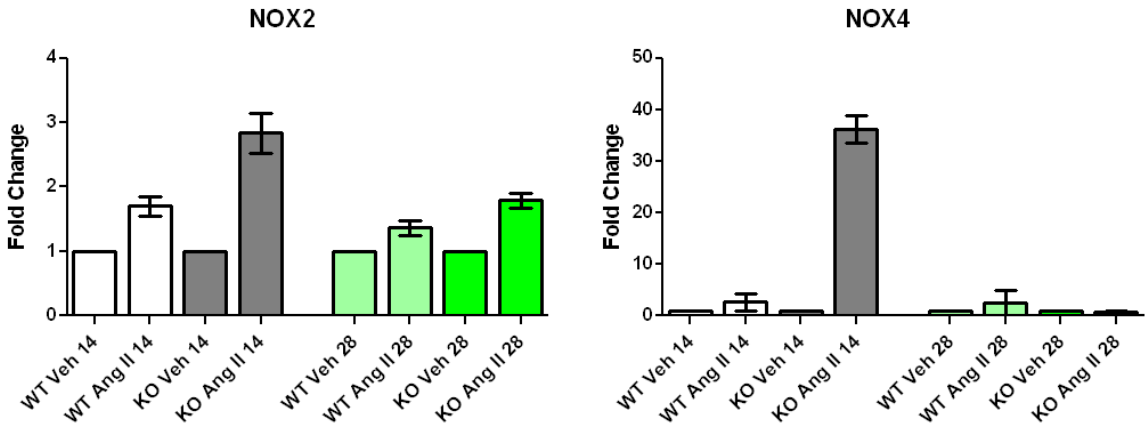


Figure 7.28. Comparing the effects of Ang II infusion for 14 days (1.1mg/kg/day) and 28 days (0.9mg/kg/day) on aortic NOX2 and NOX4 mRNA expression in WT and αCGRP KO mice. *Schematic representation of the average fold change in αCGRP and βCGRP expression in WT and αCGRP KO mice when compared to the average vehicle treated control expression after either 14 or 28 days Ang II infusion.*

In both the 14 day and 28 day study, after vehicle infusion there was no significant difference in NOX2 mRNA expression when comparing WT and αCGRP KO mice. In the onset of hypertension, after 14 days Ang II infusion, NOX 2 expression is upregulated in both WT and CGRP KO mice; however αCGRP KO mice show a larger increase in NOX2 expression in comparison to hypertensive WTs (Figure 6.4a). This trend is also mirrored after 28 days Ang II, however the fold change in NOX2 expression in the hypertensive mice is sufficiently lower than that of 14 days and there is no longer a difference in expression between hypertensive WT and αCGRP KO mice (Figure 7.19a).

When comparing the NOX4 mRNA results at day 14 and day 28, again there is no difference in NOX4 mRNA expression between vehicle treated WT and αCGRP KO mice. After 14 days, NOX4 expression did not change in WT mice after Ang II infusion (Figure 6.6a). This is also similar in WT mice after 28 days Ang II infusion (Figure 7.20a). When comparing the data for the αCGRP KO mice, after 14 days Ang II infusion NOX4 mRNA expression was significantly increased in the onset of

Ang II when compared to both vehicle treated mice and Ang II treated WTs (Figure 6.6a). This increase was approximately 35 fold when compared to the αCGRP KO vehicle treated controls. This result suggested a relationship between CGRP and NOX4 in the onset of Ang II induced hypertension. However after 28 days this increase was no longer observed, and there was no difference in NOX4 mRNA expression between vehicle and Ang II treated αCGRP KOs (Figures 7.20a and 7.28).

In this model of hypertension, we showed Ang II to result in a rise in NOX2 mRNA expression in the aorta after 14 days. This fits in with that of findings by Lassegue and Clempus and Park *et al.* who showed NOX2 expression to be elevated in the aorta of SHRs, salt-induced rat models of hypertension and in mice treated with Ang II (Lassegue and Clempus, 2003; Park *et al.* 2008). In terms of NOX 4 expression, inconclusive findings have been reported in the Ang II induced model of hypertension where some studies have shown increased NOX4 mRNA expression in hypertensive WTs (Wingler *et al.*2001; Mollnau *et al.* 2002), however others have reported decreased NOX4 mRNA expression in WT mice (Lassegue *et al.* 2001). Here we showed a slight increase in aortic NOX expression in WT mice at day 14 following Ang II which is similar to that shown by Matsuno *et al.* 2005.

This NOX expression was however significantly more elevated in hypertensive CGRP KO mice at day 14. When reviewing the literature, CGRP has previously been shown to downregulate NADPH oxidase expression and oxidative stress in Ang II-induced endothelial progenitor cells (Zhou *et al.* 2010). This result has also been shown in the ischaemia-reperfused C57BL/6 mouse intestine treated with Ang II inhibitors and CGRP via intravital microscopy studies (Yusof *et al.* 2007). Cultured rat aortic VSMCs treated with Ang II showed markedly elevated ROS production through activated NADPH oxidase. However this increase was attenuated by pre-treatment with $CGRP_{8-37}$ (Liu *et al.* 2006). These results add to the increasing evidence that CGRP is protective in the onset of hypertension, possibly via the activation of the NADPH oxidase pathway and fit in with the increasing NOX expression found in the αCGRP KO mice at day 14 in our study.

When observing the aortic expression of NOX2 and 4 at day 28 however the results were very different. NOX 2 expression was still elevated in hypertensive

mice however there was a reduction in fold change compared to that observed at day 14. This was also similar for NOX4 expression. The increased NOX2 expression was also reduced at day 28 in αCGRP KO mice, and the dramatic elevation in NOX4 mRNA expression at day 14 was no longer apparent. This suggests that the increase in NOX expression in this model is time-dependent and transient. There is little other evidence to compare with, however a study by Matsuno *et al.* (2005) used a subpressor dose of Ang II (0.14mg/kg/day) for 28 days in C57BL/6 mice to induce vascular hypertrophy and their results support our theory. This was a study looking at the involvement of NOX1 in hypertension, however they also measured the time course expression of the NOX 2 and 4 isoforms in the aortas of WT mice. They showed that during the course of Ang II infusion, expression of both NOX2 and NOX4 increased dramatically before peaking at day 7 and then decreasing again as shown in Fig 7.29. It is not mentioned what the levels of expression are by day 28, however judging by the graphs we would expect them to be of low values. This may perhaps be similar to what is happening in our model of hypertension. We see elevation in the NOX isoforms at day 14 in both WT and αCGRP KO mice; however this expression is lost by day 28. Matsuno *et al.* showed the NOX expression to peak at day 7 following Ang II infusion, therefore strongly suggesting that the role of NOX in Ang II induced hypertension is a relatively early phenomenon and we may have perhaps have even missed the peak by 14 days, although we still observe elevated expression at this stage. The dramatic elevation of NOX4 observed in the aortas of αCGRP KO mice at day 14 suggested an interactive role for αCGRP, collagen III and NOX4 in the onset of Ang II induced hypertension. If this is the case then this relationship is early in the progression of this disease, and does not play a long term role in the established hypertension as no correlation is apparent between collagen III and NOX4 at day 28.

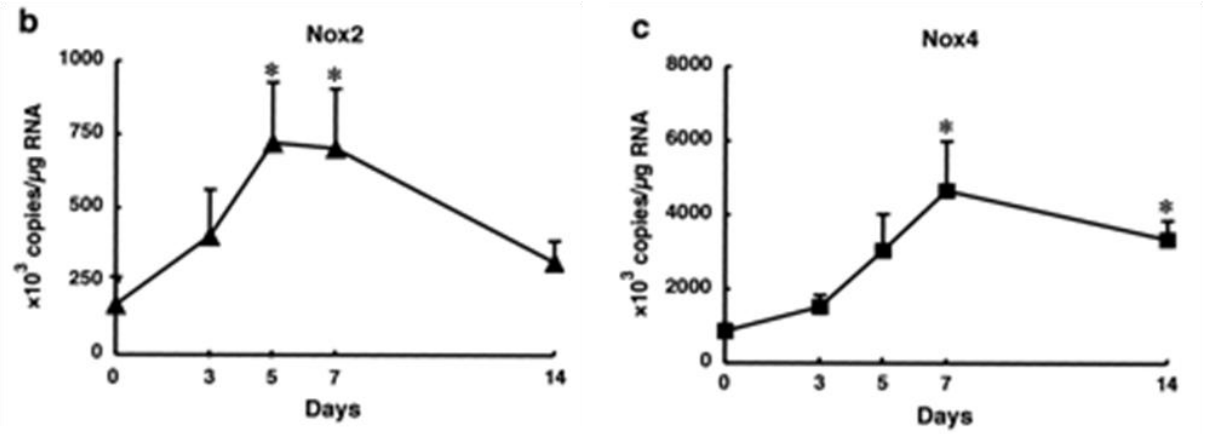


Figure 7.29 Time course of NOX2 and NOX4. Image taken from Matsuno *et al.* (2005) showing the time course expression of NOX 2 and 4 in the aortas of WT C57BL/6 mice infused with Ang II.

7.8 Limitations of this study

It is important to point out 2 noteworthy limitations of this 28 day infusion study as it may affect the direct comparison to the results shown in the previously shown results obtained at 14 days. One of these limitations is the difference in the dose of Ang II used compared to that of the 14 day study. Here we used 0.9mg/kg/day in comparison to the previously used 1.1mg/kg/day for 14 days. The simple reason for the difference in doses used is because we were unsure as to whether the mice would survive the chronic dose for 28 days, therefore it was lowered. If this study were to be repeated, we would change this and keep both studies at the same infusion dose so that direct comparisons can be made. Another point to note is with reference to the RT-qPCR data. When observing the individual graphs from the 14 day study and comparing these results to that of the 28 day study, there are considerable variations in copy numbers. At first we could have presumed it was due to the different time points measured, however when we observe more closely there are significant differences in expression of the vehicle treated groups at both time points. This could be due to a number of factors. One explanation may be because prior to this study, RT-qPCR was not developed within this research group and a significant period of my PhD was dedicated to setting up this technique within the lab. The 14 day study was conducted first; therefore this data was obtained at an earlier stage of my development in comparison to the results obtained later on for the 28 day study. Therefore the different levels of experience may perhaps be a primary reason for the difference in copies obtained. Another possibility may be due to the RT-qPCR being carried out at different times. Ideally all samples should be extracted for RNA, reverse transcribed and run at the same time. This is to ensure that the efficiency of the PCR is maintained at a constant level and so that all experimental conditions are kept the same. The standard curves which we fit our expression data to will differ with every run, and standards are also known to degrade over time. This fits with some of our data as copy numbers are lower at 28 days than that previously shown at 14 days, which may be due to degradation of standards over the time period between the two studies. When collecting samples for gene expression, it is important that they are collected with care and stored correctly in RNA later for a minimal amount of time before being extracted in order to maintain a good level of RNA quality. As explained earlier, this level of care may not have been as high

in the earlier 14 day study compared to the 28 day study simply due to a lack of experience during the earlier parts of the PhD. Samples were not extracted as quickly in the 14 day study compared to the 28 day study which may have affected the quality of the RNA. However in terms of doing all the RT-qPCR at the same time, this was not viable in this study. It was not until we obtained all the results for the 14 day study that it was decided that it would be beneficial to look at the same markers at 28 days and do a comparative study, therefore it was impossible to run all the gene expression studies at the same time. With this in mind, in order to make a good comparison of both studies, all results were transformed and expressed as a fold change in comparison to the mean expression of their matched vehicle control at each time point. This therefore allowed an accurate comparison to be made between the two studies by comparing the fold changes.

7.9 Conclusion

In this chapter I have shown that after 28 days Ang II infusion (0.9mg/kg/day) both WT and αCGRP KO mice develop sustained hypertension by day 28, and again the αCGRP KO mice displayed exacerbated hypertension when compared to hypertensive WT mice which was previously shown in the 14 day study. Aortic hypertrophy was also apparent in hypertensive mice, marked by increased collagen expression and inflammatory markers. This hypertrophy is progressively worsened in αCGRP KO mice, similar to what was previously shown at 14 days. At 28 days there is now an elevation of ET-1 mRNA expression in the aorta of hypertensive mice which was not observed previously at 14 days. At 28 days aortic eNOS mRNA expression was reduced further when compared to 14 days. These two markers indicate established endothelial dysfunction in both WT and αCGRP KO mice by 28 days of Ang II infusion. When looking at markers of the antioxidant defence system, aortic SOD1 mRNA expression continued to increase in both hypertensive groups of mice. However when looking at HO-1 and GPX expression, although these markers continue to increase in Ang II infused WT mice at day 28, this was no longer apparent in αCGRP KO mice. These markers were significantly elevated at day 14, but by day 28 the expression was lower than that of their matched vehicle controls. This suggests a depleted antioxidant defence system in the αCGRP KO mouse by 28, which correlates with the exacerbated BP increase and vascular hypertrophy. This is also coupled by an increase in oxidative stress markers such as NOX2 which is again elevated in hypertensive WT and αCGRP KO mice at day 28. However when observing aortic NOX4 expression at the 28 day time point, the significant increase in NOX4 expression previously observed in αCGRP KO mice at 14 days was not apparent anymore at day 28. This finding may well suggest that the elevation of NOX4 in the absence of αCGRP previously observed at day 14 is short lived, and therefore cancels out our previous suggestion that αCGRP and NOX4 may play an intimate and pivotal role together in the development of hypertension. The primary reason behind this time course study was to identify whether αCGRP plays a long term role in hypertension or not. We showed previously that αCGRP expression was elevated in WT mice at day 14 following Ang II infusion. In the 28 day study, when we look at αCGRP mRNA and protein expression in WT mice, this neuropeptide is still continuously increasing at day 28 to presumably try and protect the

vasculature against the progressing hypertension and end point vascular damage, thus answering our primary thoughts and confirming that αCGRP does act in a longer term in the progression of this disease. These results therefore have again supported a positive and potentially therapeutic role for αCGRP in protecting against Ang II stimulated hypertension and the progression of end organ hypertrophy.

CHAPTER 8- GENERAL DISCUSSION

8.1 Major and Novel Findings

The data obtained in this study is novel in that it has shown for the first time that αCGRP KO mice are more susceptible to the development and severity of hypertension induced by Ang II at both 14 and 28 days. In the absence of αCGRP, Ang II infusion resulted in a marked increase in arterial BP and development of severe hypertension compared to the mild-moderate hypertension developed in WT mice. This elevated BP was also accompanied by an increased inflammatory and oxidative stress profile in addition to severe end point aortic remodelling when compared to WT mice as illustrated in figure 8.1. I have also shown aortic αCGRP and receptor expression to be markedly increased in hypertension at 28 days, therefore providing evidence of a non acute presence for αCGRP in Ang II induced hypertension. This novel data provides evidence for a protective role for αCGRP in both the onset of Ang II hypertension and the longer term model of hypertension, thus making it of particular therapeutic importance in the treatment of the human disease. However the results suggest a complex series of links in terms of proposed mechanisms.

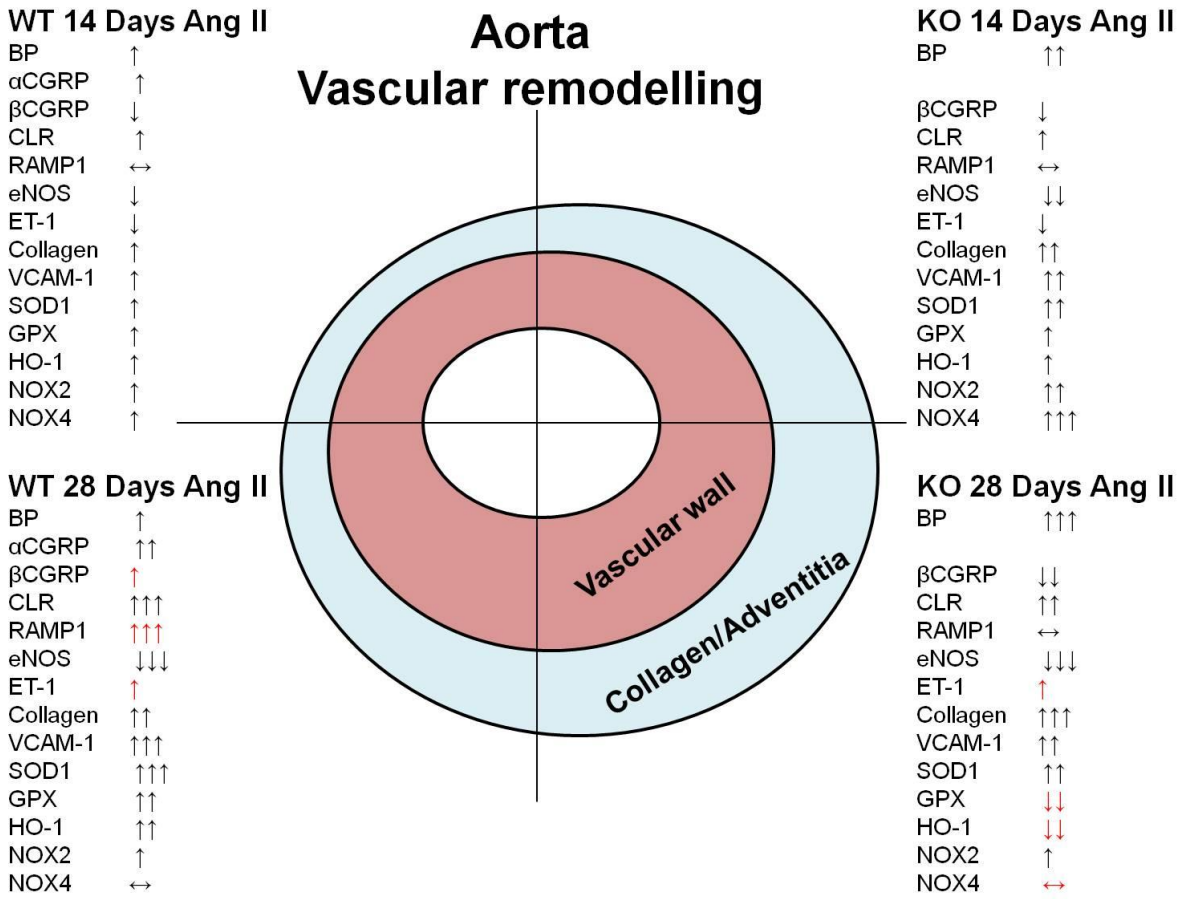


Figure 8.1 Vascular remodelling and gene expression in the aorta. Schematic diagram illustrating the vascular remodelling and gene expression which occurs in the aortas of WT and αCGRP KO mice treated with Ang II for either 14 days (1.1mg/kg/day) or 28 days (0.9mg/kg/day). Arrows highlighted in red indicate a change from the trend observed at 14 days.

8.2 Relevance to Human Hypertension

The results found in this study may provide important implications in the treatment of essential hypertension in humans, and the progressive development of cardiovascular disease in response to an elevation in BP. The Ang II experimental model utilised in this study is considered to be representative of the development of essential hypertension in humans as previously described in the general introduction. This is due to the similar response of hyperactivity of the renin-angiotensin system and the resulting increase in BP which in turn triggers the onset of damage to the vasculature via an increase in both the inflammatory profile and oxidative stress, and the onset of endothelial dysfunction and a compromised antioxidant defence system. The findings in this thesis will be discussed in terms of their relevance to the human hypertension and how this study may contribute to the development of therapeutic treatment of this disease.

As discussed previously in the general introduction (Chapter 1), hypertension remains a major public health problem and is an established risk factor for the manifestation of atherosclerosis and cardiovascular diseases which is currently the number one killer in the Western world. The high prevalence of this condition and its impact on the cardiovascular system justify it as being high priority for its detection and control, in addition to the need for further research in identifying the mechanisms behind this polygenic disease in order to develop better therapeutic targets. In the UK alone, one in three adults have high BP, and nearly half of them do not even realise they have the condition, or are not receiving any treatment (British Heart Foundation 2011).

The results provided in this thesis may suggest that the successful timing of CGRP upregulation via the administration of gene therapy or exogenous administration may be beneficial in preventing the development, or delaying the onset of the human essential hypertension. The results and their potential therapeutic value are now discussed in turn.

8.3 The Role of αCGRP in Angiotensin II-induced hypertension: Potential relationships and mechanisms and comparison of these findings to that shown in the literature published.

8.3.1 BP regulation

The vasodilatory and depressor effects of CGRP in BP under both normal physiological conditions and in the onset of hypertension has been studied extensively over the years and is therefore well documented (For review see Smillie and Brain, 2011). With regards to its role in basal BP, Ando and co workers were amongst the first to show that IV and systemic administration of CGRP into rodents causes hypotension in normotensive animals (Ando *et al.* 1990, Gardiner *et al.* 1991). In the onset of hypertension, CGRP release has been described as a key compensatory mechanism against elevating BP in both human and experimental models, suggesting a protective role for the neuropeptide in cardiovascular disease (Watson *et al.* 2011, Li and Wang, 2005, Deng and Li, 2005). These studies have been extensively reviewed in chapter one.

The development of the αCGRP KO mouse has also enhanced our knowledge of a role for CGRP in hypertension. As already discussed in the introduction, different KOs have been developed, one which is αCGRP specific and has no difference in basal BP, and a combined calcitonin/αCGRP KO which has significantly increased BP under basal conditions. However although these mice have an increased BP, they do not display any morphological changes under normal physiological conditions as previously shown by Bowers *et al.* (2005). The mice used in this thesis were the selective αCGRP KO, originally generated by Salmon *et al.* (1999). These mice were chosen as based on our understanding of the role of CGRP in humans, this would appear important for the modelling of the role of CGRP in human hypertension.

Here we have shown that at baseline, BP does not differ between WT and αCGRP KO mice, as previously described by Salmon *et al.* (1999). When we infuse mice with Ang II, hypertension develops after 14 days. However, as expected, the loss of αCGRP in the KOs results in exacerbated hypertension as previously described by Bowers *et al.* in the DOCA-salt model (Bowers *et al.*

2005). When we prolonged this infusion for 28 days to gain a model of established hypertension, BP in WT mice at day 28 did not differ significantly to that at day 14. This has also been shown previously in WT mice in a study by Jennings *et al.* (2012) using C57BL/6 mice to investigate the role of cytochrome P450 1B1 in the development of Ang II-induced hypertension over 13 and 28 days (Jennings *et al.* 2012). However in the absence of αCGRP in our study, BP continued to rise over the whole 28 day period. Based on the literature discussed, the BP results which we have observed at 14 days in this study are not surprising, however what is novel is that the hypertension is still exacerbated in the KO mice at day 28 compared to the WTs, suggesting that the protective role of αCGRP may not be acute. The data we provide in this thesis supports that previously published, and supports the hypothesis that αCGRP plays a protective role in the development of hypertension. However this is the first report of an exacerbated hypertensive response in the αCGRP KO mouse following Ang II infusion.

8.3.2 αCGRP, βCGRP and the CGRP receptor expression

As discussed previously in Chapter 4, it has been reported that the majority of human studies report decreased circulating plasma CGRP levels in patients with essential hypertension compared to normotensive controls (Portaluppi *et al.* 1992; Tang *et al.* 1989; Wang *et al.* 2007). Other studies however reported increased CGRP levels (Lind and Edvinsson, 2002; Masuda *et al.* 2002). Low to normal levels of CGRP has been observed in acute hypertension (Edvinsson *et al.* 1989), whilst severe hypertension results in a dramatic elevation in circulating CGRP in correlation with the elevated BP (Edvinsson *et al.* 1992). On the other hand, some have reported CGRP to even be unchanged in hypertension (Schifter *et al.* 1991). In this study I have shown that Ang II infusion resulted in a trend to an increase in circulating plasma CGRP levels in WT mice at both 14 and 28 days. αCGRP mRNA expression was also raised in the tissue of hypertensive mice, particularly the DRG which is considered to be the primary source of CGRP synthesis, however the role of the βCGRP isoform in these tissues was unclear and may perhaps be tissue specific. This data supports that of Portaluppi *et al.* (1993) who reported a dose dependent increase in plasma CGRP levels, in parallel with an increase in BP in response to Ang II infusion in humans (Portaluppi *et al.* 1993).

Based on the published evidence, we suggested that the role of αCGRP in the pathophysiology of hypertension may perhaps be time dependent, with αCGRP release being enhanced early in the process in order to counteract the increase in BP. Our data is novel in that we have shown αCGRP expression not to be time-dependent in this Ang II-induced model whereby circulating plasma CGRP and αCGRP vascular and neuronal tissue mRNA expression continued to increase as the hypertension progressed from days 14 through to 28.

It was originally thought that βCGRP was only expressed in the enteric nerves, however Holmann *et al*. reported βCGRP to be expressed and act as a functional vasodilator in the coronary vessels of hearts isolated from rats and rabbits after perfusion with vasopressin (Holmann *et al.* 1986). We support this finding in our model whereby we have identified βCGRP mRNA and protein expression in the vasculature, including the aorta. We have also reported this mRNA expression to be significantly more abundant in all tissues than αCGRP, including the DRG, which does not support previous findings by Schutz *et al.* who reported αCGRP gene expression to be the most abundant isoform in WT mice when comparing α and β CGRP expression patterns in WT and αCGRP KO mice under basal conditions (Shutz *et al.* 2004). This βCGRP mRNA expression also continued to increase in the aortas of WT mice from day 14 to 28, independently of an increase in BP. This may perhaps be related to an increase in CLR/RAMP1 mRNA expression. The loss of βCGRP in the hypertensive αCGRP KO mice was unexpected as it was originally hypothesised that perhaps this isoform would compensate for the lack of αCGRP. A possible reason for this loss though may be linked to neuronal degeneration in the mice as the hypertension progresses. Exacerbated oxidative stress and antioxidant abnormalities in hypertension are key markers of neuronal degeneration (Rao *et al.* 2002). We have observed these signs in the KO mice as hypertension progresses, thus providing a possible reason for the loss of βCGRP mRNA expression in tissues at day 28. Overall though, the high abundance of βCGRP expression found in this model suggests that this isoform may be more important in vascular disease than originally anticipated, which therefore opens up a whole new avenue in understanding the role of CGRP in vascular diseases.

Supowit and co workers have successfully studied the role of CGRP in the rat previously in which they report that CGRP acts as a depressor mechanism and attenuates BP increases in three models of hypertension: DOCA-salt (Supowit *et al.* 1997), SN-salt (Supowit *et al.* 1998) and L-NAME (Gangula *et al*. 1997). In the DOCA-salt model they showed that the antihypertensive effect of CGRP was mediated by an upregulation of neuronal CGRP expression and release, however in the SN-salt model this depressor effect was independent of a change in CGRP expression. They concluded that instead, the vasculature may be hyper responsive to the vasodilator activity of CGRP. In a study to assess this matter, they again used a model of SN-salt hypertension and concluded that the depressor effect of CGRP was indeed due to increased sensitivity of the vasculature in hypertensive rats compared to controls, and the mechanism was via a significant increase in receptor component protein (RCP) expression but no change in CLR or RAMP1 expression (Supowit *et al.* 2011). This result was supported by others which also showed that the vasodilator regulatory effects of CGRP are regulated at the level of the vasculature via mechanisms involving alterations in the CGRP receptor expression (Hasback *et al*. 2005; Kawai *et al*. 2002; Ma *et al*. 2003; Prado *et al.* 2002; Thota *et al*. 2003). These studies show that enhanced responsiveness to CGRP is mediated through an increase in RCP. RCP is reportedly pivotal in CLR/RAMP1 functioning *in vivo* (Supowit *et al.* 2011), with reports also showing that a decline of RCP expression also caused a marked decline in CGRP expression during parturition of the pregnant mouse independent of any changes of CLR/RAMP1 expression.

In this study I have shown that in WT mice, at day 14 αCGRP mRNA expression was increased, with slight increases in CLR but not RAMP1 mRNA expression, however by day 28, αCGRP mRNA expression continues to rise, and this rise is also in accordance with an elevation in both CLR and RAMP1 expression. At this time-point, αCGRP plasma protein content was also upregulated. This data may suggest that the increase in αCGRP is dependent on an increase in receptor expression. On the other hand, when we look at the receptor mRNA expression in the αCGRP KOs, the results indicate a different mechanism. Surprisingly, βCGRP mRNA expression was downregulated in the aorta, upregulated in the heart, but not changed in the kidney, MRV or DRG at both 14 and 28 days following Ang II infusion. Based on this result alone, we are unable to tell whether βCGRP plays a

compensatory role for the loss of αCGRP in these mice. This role may perhaps be tissue specific. If we focus on the aorta, this downregulation was independent of a change in CLR/RAMP1 gene expression. RCP gene expression was not measured here, however based on this result it would be beneficial to observe this expression in future studies based on the evidence provided in the literature and comparing that to the results presented here.

8.3.3 Endothelial dysfunction, the antioxidant defence system and onset of oxidative stress and vessel inflammation /remodelling

eNOS

At day 14, aortic eNOS expression was decreased in the aortas of WT and αCGRP KO mice in response to the elevated BP. This reduction in eNOS has also previously been reported in humans in the development of hypertension and increased BP (Uren *et al.* 1992). When we prolonged this study to 28 days, this eNOS expression decreased further, which supports findings by Gu *et al.* who showing eNOS expression to decrease in a time-dependent manner as hypertension progresses and the vasodilatory mechanisms are lost (Gu *et al*. 2010). At day 14 eNOS expression in αCGRP KO mice was significantly lower than that of WT mice in the onset of Ang II induced hypertension, however no difference was observed in the established hypertension at 28 days. This result suggests that either a) αCGRP is protecting against the hypertension progression, or b) αCGRP is protecting against the loss of NO at day 14, however this mechanism is lost by day 28, therefore this may be a time-dependent mechanism/relationship. It is also possible that the increase in BP is due to a result of the loss of NO derived from eNOS, and not just, or even in part from the vasodilator CGRP. The results do emphasise though that the reduction in eNOS expression is one of the early mechanistic changes.

ET-1

At day 14 plasma ET-1 levels did not differ between the 4 groups, and aortic gene expression suggested ET-1 to be downregulated in the hypertensive animals. When the Ang II infusion was prolonged however, this trend in the aorta changed

and ET-1 appeared to be upregulated, although not significantly. The lack of significant changes in ET-1 expression in this model was unexpected, and does not support findings of elevated ET-1 expression reported previously in this model of hypertension (Rajagopalan *et al.* 1997). In addition to this, the lack of difference in ET-1 expression between WT and αCGRP KO mice suggests that αCGRP does not play a role in ET-1 expression in this Ang II induced model of hypertension. This does not support studies which have proposed a direct interaction between ET-1 and CGRP primarily due to ET_A receptors being expressed on sensory motor nerves alongside CGRP (Wang *et al*. 2006), and ET-1 induced arterial contractions are reversed and therefore sensitive to relaxation by the administration of CGRP (Meens *et al*. 2009; Meens *et al.* 2011). ET-1 and CGRP expression has also been reported to be elevated in cardiac and renal tissue in the SHR (Nelissen *et al.* 2011). Our findings may either suggest that this interaction is model specific, and not involved in Ang II-induced hypertension, or else perhaps the non-significant upregulation observed in the aorta may suggest that the upregulation of ET-1 in this model is not at an early stage. It would have been beneficial to also observe the plasma ET-1 levels at the 28 day time point. Overall these results suggest that αCGRP is acting independently of ET-1-dependent mechanisms at the 14 day time point at least.

Antioxidants

In the onset of Ang II induced hypertension, the decrease in endothelial NO bioavailability observed in our study may well be related to the upregulation of the antioxidant defence system (SOD1, GPX, and HO-1) as measured by RT-qPCR. This result was not surprising as increased expression of these enzymes have been reported previously in WTs in the Ang II-induced model of hypertension (Vera *et al.* 2007; Yang *et al.* 2004; Wang *et al.* 2002). As previously discussed in Chapters 6 and 7, these enzymes are key mediators in the onset of hypertension where they play an important role in the scavenging of oxidative superoxide radicals produced in response to elevated BP, Ang II upregulation, and a reduction in NO bioavailability. The use of global knockout mouse models have successfully shown deletion of either SOD1, HO1 or GPX to result in exacerbated hypertension marked by higher BP and more profound end organ damage when compared to WTs following Ang II infusion, therefore it is already established that these antioxidants play a pivotal and protective role in this experimental model

(Hu *et al.* 2004; Ardanaz *et al.* 2010; Carlstrom *et al.* 2010). Based on our results in the WT mice, our work fits with that of the literature previously discussed. However, this antioxidant expression is significantly elevated in the absence of αCGRP at day 14. This result is expected due to it fitting with the exacerbated BP and greater degree of eNOS reduction at this stage of the hypertension. However it is striking is that the antioxidant expression in these mice is then compromised at day 28, which is also accompanied with a complete loss of eNOS expression. This reduction in antioxidant levels as hypertension further progresses fits with that previously shown by Alba *et al.* and Das, 2006, as previously discussed in Chapter 7. As already mentioned previously, the further increase in GPX expression whilst SOD and HO-1 decreases in these mice is unclear, however Wang *et al.* also reported similar results in pre-hypertensive patients with decreased CGRP levels compared to normotensive subjects, with no previous literature to support this, and therefore makes our study the first to support these findings (Wang *et al.* 2007). Thus the endogenous anti-oxidant gene expression is lost relatively soon and by day 28 any role of αCGRP in protecting has diminished.

Vessel inflammation and remodelling

It is also well documented that CGRP is cardioprotective and can potentially inhibit end-organ damage in hypertension. Administration of $CGRP_{8-37}$ increased cardiovascular damage in the salt and L-NAME-induced hypertensive rats (Supowit *et al.* 1997; Bowers *et al.* 2005; Wang *et al.* 2006; Gangula *et al.* 1997). In addition to this, αCGRP/calcitonin KO mice show increased susceptibility and blunted recovery to ischaemia/reperfusion injury characterised by increased vascular cell damage and ROS generation (Huang *et al.* 2008). This blunted recovery in ischaemia was also observed in diabetic mice which were unable to generate cardioprotection due to the loss of αCGRP activity. However CGRP administration into these rodents was shown to rectify this (Zheng *et al*. 2011). In the Ang II model, over expression of RAMP1 protected against Ang II –induced endothelial dysfunction via the elevation of CGRP production (Chrissobolis *et al.* 2010).

Studies *in vitro* have also shown that CGRP inhibits rodent cardiomyocyte cell apoptosis after induction of norepinephrine (Zhao *et al*. 2010). In cultured

endothelial cells, CGRP greatly reduced the amount of migrating neutrophils, monocytes and chemokines to the endothelium via the inhibition of NFκB, therefore expression of inflammatory markers was reduced (Huang *et al*. 2011).

Vascular hypertrophy is considered to be an adaptive response to increased arterial wall stress in hypertension, (Obayashi *et al.*1999) and it has been well documented that Ang II induces vascular remodelling through both direct effects of BP and indirectly through vascular inflammation (Liao *et al.* 2008). When designing this study, a vast literature search was first conducted to find out which markers of inflammation and oxidative stress were typically upregulated in the Ang II-induced model of hypertension. We then measured these markers in WT mice at 14 and 28 days following Ang II infusion to compare it with the already published literature. Our data fits in with that previously shown in that our WT mice showed enhanced vascular inflammation and oxidative stress marked by increased expression of markers including VCAM-1, IL-6, NOX2 and collagen III. TGF-β expression, which is a marker of vessel remodelling was not increased in the aorta, but was increased in the DRG. When observing aortic morphology, 14 days Ang II infusion stimulated remodelling of the vessel characterised by increased wall width and collagen deposition. Expression of these markers and vascular remodelling continues to increase in these mice when Ang II infusion is prolonged, independently of an increase in BP which supports the findings by Jennings *et al.* (2012) who also reported enhanced inflammation and remodelling at day 28 following Ang II compared to that observed at day 13, although no further increase in BP was observed (Jennings *et al.* 2012).

With the results of the WT mice fitting in with that already previously published, we were than able to identify a role for αCGRP in this model. When referring back to the DOCA-salt study by Bowers *et al*. they showed the elevation of BP in the calcitonin/αCGRP KO to be accompanied by an increase in end-point renal organ damage and elevated VCAM-1 and other pro-inflammatory mediators when compared to the inflammation observed in the DOCA-salt treated WTs (Bowers *et al.* 2005). This is a key paper to compare our research to as we have also observed similar findings in our study. We have reported here for the first time that αCGRP mice display exacerbated vascular inflammation, oxidative stress and vascular remodelling at day 14 following Ang II infusion when compared to WT mice, characterised by marked increases in VCAM-1, Collagen III, IL-6 and

NOX2. Aortic remodelling was significantly worsened in these mice. What was particularly striking was the significant increase in NOX4 expression in the aorta, which was found to be localised in the adventitia and collagen layer of the aorta. NOX4 derived ROS has been reported previously to be involved in vessel remodelling, whereby it was shown to be increased in mesangial cell hypertrophy in response to Ang II (Gorin *et al.* 2004). In addition to this, NOX4 has been located in human pulmonary arterial SMCs, primarily to the perinucleus, and is involved in cell proliferation (Mittal *et al*. 2007). NOX4 expression in these pulmonary vessels and arteries is significantly elevated in patients with pulmonary hypertension (Mittal *et al.* 2007; Hecker *et al.* 2011). When we put the data from this study together in a correlation graph we showed that there was a direct correlation between collagen expression and NOX4 expression in the αCGRP KO mice following 14 days Ang II infusion, therefore suggesting an early relationship between αCGRP, NOX4 and collagen formation in this model, which may elucidate a key protective mechanistic role for this vasodilator. The data provided in our study at 14 days supports the hypothesis that αCGRP has an inhibitory effect on the proliferation of VSMCs and plays a protective role in Ang II induced hypertension. However, the mechanism underlying this protective effect of is unclear. CGRP perhaps may have reduced the viability and DNA synthesis of VSMCs, and thus decreased the increased proliferation index of VSMCs and collagen formation induced by Ang II. It has been reported that CGRP inhibits the proliferation of VSMCs in conjunction with an elevation of the second-messenger, cAMP (Li *et al.* 1997).

In the established model of hypertension following 28 days Ang II infusion, markers of inflammation and oxidative stress are still raised; however this expression is no longer exacerbated in comparison to the WTs. It is unknown why this is the case, with no evidence to support this in these knockout mice, however this may possibly be due to the hypertension now being fully established in these mice at day 28, or else connected to the loss of the antioxidant defence system. The significant rise in NOX4 was also no longer apparent. Matsuno *et al*. (2005) have previously shown that NOX expression after Ang II infusion is time-dependent and peaked at day 7, therefore the time point and progressive state of the hypertension may be the reason for this decreased expression of these markers. However if this is the case, it is of question why the BP continues to rise,

and could this rise in blood pressure now be dependent on other mediators instead. In addition to this, the dramatic elevation of NOX4 observed in the aortas of αCGRP KO mice at day 14 suggested an interactive role for CGRP and NOX4 in the onset of Ang II induced hypertension; however this elevation was diminished at day 28. This suggests that this potential relationship between NOX4 and αCGRP is early in the progression of this disease, perhaps key in the onset of the hypertrophy response, but does not play a long term role in the established hypertension.

8.4 Potential therapeutic roles of CGRP

Excessive CGRP-mediated vasodilatation has been associated with the pathogenesis of migraine, and as such the development and use of CGRP antagonists have been proposed in its treatment (Juhasz *et al.* 2003; Goadsby *et al.* 1990). Subcutaneous injection of BIBN4096BS in migraine sufferers causes reduction of symptoms without affecting blood pressure in these normotensive migraine sufferers (Olesen *et al.* 2004 and Russo, 2007) and the most recently identified CGRP antagonist Telcagepant (Merck) has also been involved in clinical trials as anti-migraine drugs, and have no adverse effect on arterial pressure (Edvinsson and Linde, 2010). The development of Telcagepant however was terminated in 2009 where it reached phase two of clinical trials due to significant elevations in serum transaminases being reported in two patients taking the drug, therefore it being posed as risk of causing hepatic damage (Clinical Trials, Gov).

However, it is important to assess the cardiovascular risk associated with administration of these CGRP antagonists as CGRP-mediated vasodilatation may be beneficial in hypertension and be cardioprotective based on the evidence shown in this thesis. It may be assumed that if migraine treatments are given on an occasional basis, when the onset of migraine is detected in a normotensive healthy patient with a relatively low incidence of attacks, then the vascular risk may be small. However, if the patient suffers more frequent attacks and the requirement of these antagonists is on a regular basis, the potential risk of adverse cardiovascular events may be more difficult to assess due to a lack of understanding of the total role of CGRP in both migraine and BP control. This problem has been questioned by a number of groups, looking first for effects of

$CGRP_{8–37}$ (Chiba *et al*. 1989) and more recently using non-peptide antagonists in animal models (Doods *et al.* 2000). It has been shown that telcagepant (MK-0974) does not induce contraction or relaxation of human coronary vessels, i.e. a neutral effect, under conditions whereas the (5-HT)1B/1D agonist triptan, also used in migraine treatment, posed an adverse effect of coronary vasospasm (Chan *et al*. 2010; Regan *et al.* 2009). These results build upon a body of evidence, suggesting that the CGRP antagonists should be safer than the triptans in mediating adverse side effects in the heart. However further studies may be warranted, based on my findings in disease models.

Based on the findings from this thesis and the previously reported literature, the use of CGRP agonists pose as an interesting therapeutic target for the treatment of hypertension and the onset of cardiovascular diseases. CGRP acts as a depressor in systemic hypertension. An increase in its expression has been shown to have a compensatory role to attenuate the elevated BP in DOCA-salt and L-NAME treated rats (Supowit *et al.* 1997; Gangula *et al.* 1997). CGRP levels are also elevated in patients with heart failure (Ferrarri *et al.* 1991), and capsaicin treated (CGRP depleted) pigs have more profound myocardial infarction and increased BP in comparison to controls (Kallner *et al*. 1998). This evidence, alongside the evidence discussed in chapter one and the results of this thesis strongly suggest a cardioprotective role for CGRP.

As such, CGRP has undergone clinical trials in a variety of cardiovascular disorders whereby vasodilatation would be beneficial. IV administration of 50µg CGRP was shown to reduce BP in essential hypertensive patients (Tang *et al.* 1989). This elevated BP was also reduced in patients with chronic stable angina compared to those treated with a placebo (Uren *et al.* 1993). Short-term IV infusion of CGRP (12.5µg/hr for 14 hours) was shown to improve myocardial contractility in patients with heart failure (Gennari *et al.* 1990). However another study showed this protective effect to be lost again within 30 minutes of stopping CGRP infusion (Shekhar *et al.* 1991), therefore suggesting that the role of CGRP is short-lived. CGRP administration (IV) has also been reported to have beneficial effects in Raynaud's disease (Shawket *et al.* 1991; Bunker *et al.* 1993) and subarachnoid haemorrhage (European CGRP in subarachnoid haemorrhage study group, 1992).

However, although the vasodilatory effect of CGRP has been shown to be beneficial in these cardiovascular disorders, its clinical potential is limited due to it not being available orally, its quick breakdown, and potential adverse side effects in response to chronic administration (Doggrell *et al.* 2001). Chronic administration could possibly lead to flushing, hypotension and fainting, in addition to the onset of migraine. Instead, studies have proposed the effects of mimicking CGRP with the use of capsaicin/vanilloid receptor agonists (normally associated with pain) which promote CGRP release. However long term capsaicin was shown to cause CGRP depletion (Alving *et al.* 1991), therefore this may not have potential in a clinical situation. Other possibilities include the use of gene therapy whereby CGRP gene delivery directly into the VSMC of the rat aorta was shown to inhibit VSMC proliferation in response to balloon injury (Wang *et al.* 2004). More recently NovoNordisk have developed a novel and stable αCGRP analogue (NNO 174-0000-0308) which possesses a substantially longer half life of ~7 hours when compared with <30mins in CGRP (Neilsen-Sams, Patent WO2011/051312 A1, issued 2011). This analogue is selective for the CGRP receptor and has been shown to be active in terms of hypotension in the rat (Nilsson *et al.* 2010). However it is still not of clinical potential due to it not being orally available. On the other hand it potentially provides an interesting pharmacological tool for murine studies. There is therefore a potential “gap” in the market for the development of an orally available CGRP agonist with a long half life for the therapeutic treatment of hypertension and cardiovascular conditions.

8.5 Limitations of the experimental study and design

At present, the two techniques commonly used to measure BP in conscious rodents are tail cuff plethysmography and radiotelemetry. Within our group, tail cuff plethysmography has been developed and used successfully on numerous occasions, (Clark *et al.* 2007). However in recent years, developments have been made in the use of radiotelemetry and this technique is now referred to as the “gold standard”, and is therefore of preference by reviewers when trying to publish research on BP measurements. The use of tail cuff to measure BP in this study and not telemetry may therefore pose as a limitation of this study. However, as already discussed previously, comparative studies have been carried out previously using parallel groups of mice to measure the accuracy of tail cuff

measurements to that of radiotelemetry (Whitesall *et al.* 2004; Kramer and Kinter, 2003). This has also been done in our group and we showed that when mice are trained correctly, there are no variations in the BP trend observed from tail-cuff plethysmography compared to radiotelemetry (Marshall *et al.* 2009), although radiotelemetry readings are slightly lower. In order to minimise any inaccuracies and false measurements, the animals used in this study underwent a strict training regime for at least 2 weeks prior to actual recordings taking place. This regime involved training the mice to allow them to become acclimatised to the potentially stressful environment. Therefore by the time the actual recordings were taken, it was ensured that the mice are calm and not stressed. The recordings were also taken at the same time every day and the surrounding environment was kept constant e.g. temperature and light controlled. However, there are still some advantages which radiotelemetry has over tail-cuff (e.g. no need to restrain the animal, can record 24hr/day) which makes it a more suitable method of recording BP in certain experimental models, and as such it would be useful to utilise this technique in this model of hypertension in future.

Another limitation is the general measurement of gene rather than protein expression. This study focuses on observing changes at the pre-transcriptional levels rather than measuring the end products by protein. Some of our positive mRNA results may not be found when measuring protein levels; therefore we may be observing changes and drawing “premature” conclusions. Probing by Western Blotting is the preferred method for looking at specific markers, however this method raises the issue of using non-specific antibodies. It is also a very lengthy process in comparison to RT-qPCR. This method is very reliable and extremely specific, and has therefore become a more popular choice by research groups. The use of the primers to target specific targets of a gene ensures minimum variability. It is not dissimilar to microarray which is becoming increasingly popular due to the ability to scan the whole genome in a very short space of time. It would also deem impossible to confirm every change observed in a microarray by protein. Here we have scanned a wide range of genes which have been previously shown to be expressed in these tissues in this model, and the preferred use of PCR and not Western Blotting has allowed us to generate a vast amount of accurate and, we believe, reliable data in a relatively short space of time. Although changes of expression at this level may not be transcribed into protein,

the measurement of gene expression at this early level may prove critical in identifying key pathways in the progression of diseases. It is noted that there is the possible variability in the RT-qPCR results.

It could also be said that the lack of identifying the mechanisms by which CGRP protects in this model of hypertension could be a limiting factor in this study, therefore a number of future experiments in order to potentially tackle the key mechanism are now proposed:

8.6 Future Work

A number of experiments are proposed in order to better understand the mechanisms behind the results shown in this thesis. The role of CGRP and its mechanisms of action are still not fully understood at this stage. Based on the results presented in this thesis, it was hypothesised that the protective effects and mechanisms by which CGRP acts occurs at a very early stage in the onset of hypertension as we see dramatic changes occurring by day 14 following Ang II infusion. However some of these changes do not continue to appear when the infusion time is prolonged. In order to investigate this early protective role it would be beneficial to carry out a 7 day Ang II infusion study in these mice and determine how early the changes are occurring in the absence of αCGRP.

It was also uncertain as to whether the increasing inflammatory and oxidative profile in addition to the vascular remodelling found in the αCGRP KO is primarily due to the lack of αCGRP or whether it is a result of the increased BP, and therefore pressure dependent. Following 14 days Ang II infusion, BP was elevated in the WT mice, this being within the borderline-mild hypertension range; however this BP increase was significantly exacerbated in the αCGRP KO mice. This result in the αCGRP KOs was also accompanied by a higher degree of aortic remodelling. When correlating this to the human hypertension, it has been shown that end organ damage is correlated to an increase in BP. Little vascular remodelling and damage may be expected in the borderline-mild hypertensive patients, whereas a larger degree of damage will be observed in patients with severe hypertension, which has been shown here in the αCGRP KO mice. Therefore it could be suggested that the degree of vascular remodelling may

perhaps be BP dependent and not a direct effect of the lack of αCGRP. However when the infusion of the Ang II was prolonged to 28 days, although there was no further increase in BP in the WT mice, there was in fact a continuing increase in vascular remodelling and inflammatory/oxidative profile, suggesting that this remodelling is a direct effect of the Ang II, and not a direct increase in BP. When prolonging the Ang II infusion in the KOs however, the BP continued to rise to a dangerously hypertensive state. The absence of this continuing increase of BP in WTs makes us suggest that this increase in KOs is a direct effect of the absence of αCGRP. Moreover many studies have been cited in this thesis where CGRP has been shown to be protective in *in vitro* cell studies.

To answer this the use of a subpressor dose of Ang II would be beneficial as these lower doses e.g. 0.2-0.3mg/kg/.day have been reported to not increase BP in mice at 14 days (Bendall *et al.* 2002; Murdoch *et al.* 2011). By using this subpressor dose in WT and αCGRP KO mice we can then determine any changes between the Ang II treated WT and αCGRP KO mice in the absence of the increased BP, and determine a more accurate role for αCGRP in this model. The use of *in vitro* studies using aortas from these mice and treating them with Ang II and then measuring the redox and inflammatory profile may also be beneficial. Other useful experiments in utilising mechanisms would be to measure cAMP levels in addition to measuring a marker of ROS production and damage such as nitrotyrosine. Results from these may provide key insight into mechanisms by which CGRP acts in a protective manner.

8.7 Summary

To summarise and conclude, the results of this thesis are novel in that they reveal a potentially protective role for αCGRP in both the onset and developed model of Ang II induced hypertension whereby deletion of αCGRP enhanced hypertension induced end organ damage. Further work is required to elucidate the mechanisms by which CGRP acts, as at this stage it is difficult to predict whether this damage is a due to a direct loss of αCGRP, or whether it is partially BP dependent. However it is hoped that the findings presented in this thesis have both advanced our knowledge of CGRP and its receptor, and can be utilised for the development of therapeutic and pharmacological treatment of hypertension.

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